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CHARACTERIZATION OF A HUMAN B LYMPHOCYTE-SPECIFIC ANTIGEN[1]

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A human B lymphocyte-specific antigen (B1) was identified and characterized by the use of a monoclonal antibody. By indirect immunofluorescence, cytotoxicity, and quantitative absorption, B1 was present on approximately 9% of the peripheral blood mononuclear cell fraction and >95% of B cells from blood and lymphoid organs in all individuals tested. Monocytes, resting and activated T cells, null cells, and tumors of T cell and myeloid origin were B1 negative. B1 was distinct from standard B cell phenotypic markers, including Ig and Ia antigen. Removal of the B1 positive population in peripheral blood eliminated all B cells capable of responding to pokeweed mitogen by maturation to Ig-producing cells.

Human B lymphocytes possess a distinct cell surface phenotype that distinguishes them from other lymphoid cell populations. These markers include integral membrane immunoglobulin (Ig) (1), receptors for C3 and the Fc portion of IgG (2, 3), and the presence of antigens encoded by the HL-A-D locus, the so-called DR antigens (4–8). The latter appear to be biochemically and functionally analogous to murine I-E/C region (Ia) antigens (9, 10).

However, because many of these phenotypic markers are not restricted in their expression to B cells, their utility in cell enumeration and fractionation is somewhat limited. Distinction between B cells and monocytes presents particular difficulties, since the latter also bear Fc and C3 receptors (11) and Ia antigen (12, 13), and may bind Ig via their Fc receptors, producing false Ig positivity (14). In addition, Fc receptors may be present on a subset of T cells (15), and T cells have been shown to express Fc receptors (16) and Ia antigens (17, 18) subsequent to activation.

A phenotypic marker with representation limited to B lymphocytes would therefore be extremely useful in the enumeration, fractionation, and analysis of function of the B cell population. Heteroantisera with B cell specificity have previously been described, produced by cross-species immunization and extensive absorption with non-B cell lines and tumors. Some of these antisera appear to define B cell-specific alloantigens (19–23), although many have been found to be principally directed against HL-A-D locus antigens (m.w. 29,000 and 34,000) (5–8, 12), and are thus also present on monocytes and other cell types (24).

In the present investigation, a monoclonal antibody specific for human B cells is described and characterized. This antibody was found to define an antigen (B1) present on approximately 9% of peripheral blood mononuclear cells (PBM)[3] and >95% of peripheral blood B cells from all individuals tested. B1 was found to be unrelated to known phenotypic markers of B cells, including Ig and known Ia antigens. Functional studies demonstrated that removal of the B1-positive population from peripheral blood by cell sorting or complement-(C) mediated lysis, eliminated the cell population that is induced to differentiate into Ig-secreting plasma cells in a pokeweed mitogen-(PWM) driven system.

MATERIALS AND METHODS

Immunization and somatic cell hybridization. A 6-week-old female BALB/c mouse (Jackson Laboratories, Bar Harbor, ME) was immunized i.p. with 5×10^6 cryopreserved Burkitt's lymphoma tumor cells in phosphate-buffered saline (PBS). Twenty-eight days later, the mouse was boosted with 5×10^6 tumor cells i.v., and somatic cell hybridization was carried out 4 days later by the method of Kohler and Milstein (25), with modifications (26). Mouse splenocytes (1.5×10^8) were fused in 30% polyethylene glycol (PEG) and Dulbecco's MEM with 2×10^7 P3/NS1/1-Ag4-1 myeloma cells (kindly provided by Dr. R. Kennett, University of Pennsylvania, Philadelphia, PA).

Selection and growth of hybridomas. After fusion, cells were cultured in HAT medium (hypoxanthine, aminopterin, and thymidine) at 37°C in a 5% CO_2 humid atmosphere (27). Fourteen to 28 days later, 100 μl of supernatant from cultures exhibiting cell growth were tested for the presence of hybridoma antibodies reactive with the immunizing Burkitt's tumor cell by indirect immunofluorescence as previously described (28). In brief, 10^6 cells were incubated with culture supernatants at 4°C for 20 min, washed twice with medium, and stained with a fluoresceinated goat anti-mouse IgG (G/M FITC) (Meloy Laboratories, Springfield, VA) for 20 min on ice. After an additional 2 washes, fluorescent antibody-coated cells were analyzed on a fluorescence-activated cell sorter (FACS-I) (Becton Dickinson, Mountain View, CA), or a cytofluorograf FC200/4800A (Ortho

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[3] Abbreviations used in this paper: CLL; chronic lymphatic leukemia; D-PDL, diffuse, poorly-differentiated lymphoma; E+, sheep erythrocyte positive; FACS, fluorescence-activated cell sorter; G/M FITC, fluorescein-conjugated goat anti-mouse IgG; PBM, peripheral blood mononuclear cells; PEG, polyethylene glycol; PWM, pokeweed mitogen; RIA, radioimmunoassay; sIg+, surface Ig positive; TCM, tissue culture medium; DMSO, dimethyl sulfoxide; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Instruments, Westwood, MA). Hybridoma cultures containing antibodies reactive with the immunizing tumor were selected and cloned by the limiting dilution method in the presence of feeder cells (29). Recloned hybridomas were subsequently maintained by injection of 1×10^6 cells i.p. into BALB/c mice primed with pristane (Aldrich Chemical Co., Milwaukee, WI). Monoclonal antibody-containing ascites were used in all subsequent experiments.

Isolation of lymphocyte populations. Human PBM were isolated from healthy volunteer donors by Ficoll-Hypaque density gradient centrifugation (Pharmacia Fine Chemicals, Piscataway, NJ) (30). Unfractionated mononuclear cells were separated into surface Ig-positive (sIg+) (B) and sIg− (T plus null) populations by Sephadex G-200 anti-$F(ab')_2$ chromatography (31), with modifications designed to minimize monocyte retention by the column. In brief, purified rabbit anti-human $F(ab')_2$ was pepsin-digested and chromatographed on Sephadex G-150 to remove undigested material. The $F(ab')_2$ anti-human $F(ab')_2$ fragments were then coupled to CNBr-activated Sephadex G-200. PBM were preincubated at 37°C for 1 hr to remove cytophilically-bound serum IgG (32), and cells were then applied to the anti-$F(ab')_2$ column and fractionated as outlined previously (31). The sIg+ (B) population was obtained from the Sephadex G-200 column by competitive elution with normal human γ-globulin. B cell preparations were routinely >90% sIg+, <5% E+, and contained approximately 5% monocytes as judged by morphology, latex ingestion, and reactivity with the monocyte-specific monoclonal antibody OKM1 (33). T cells were recovered by E rosetting the sIg− population with 5% sheep erythrocytes (Microbiological Associates, Bethesda, MD). The rosetted mixture was layered over Ficoll-Hypaque, and the recovered E+ pellet was treated with 0.155 M NH_4Cl to lyse erythrocytes. The T cell population obtained was <2% sIg+ by methods previously described (34). Normal human monocytes were obtained by adherence to plastic culture dishes as previously described (28). For determination of the reactivity of anti-B1, 1×10^6 of each cell population was subjected to indirect immunofluorescence analysis as described above.

In experiments designed to separate B1+ and B1− cells, 100×10^6 unfractionated PBM were labeled with 4 ml of a 1/100 dilution of anti-B1 and developed with G/M FITC. Cells were then separated on a FACS-I into B1+ (8.3% of PBM) and B1− populations. All fractions were washed 3 times and placed into culture for functional studies.

Normal human tissue. After appropriate Human Protection Committee validation and informed consent, human specimens were obtained during surgery. Nucleated bone marrow cells were recovered by Ficoll-Hypaque centrifugation. Tonsil cells were obtained at the time of routine tonsillectomy. Lymph node tissue was taken for diagnostic biopsy, and was considered normal based on histology and cell surface markers. Splenocytes were obtained at the time of traumatic rupture. Normal human thymocytes were obtained from patients who had portions of their thymuses removed during corrective cardiac surgery. All tissue specimens were immediately placed in media containing 5% fetal calf serum (FCS), finely minced with forceps and scissors, and made into single-cell suspensions by extrusion through stainless steel mesh. Cell samples were cryopreserved and thawed as needed.

Cell lines and leukemia and lymphoma tumor cells. Epstein-Barr virus- (EBV) transformed B lymphoblastoid lines (SB, Laz 007, 234, 296, 388, and 444), T cell lymphoblastoid lines (CCRF-CEM and HSB-2), Null cell leukemia line Laz 221, and Burkitt's lines Daudi, Ramos, and Raji were kindly provided by Dr. Herbert Lazarus (Sidney Farber Cancer Institute, Boston, MA). Tumor cells were obtained from 19 patients with acute and chronic leukemias, and 12 patients with various forms of non-Hodgkin's lymphomas. In all instances, the tumor populations utilized contained more than 90% blasts by Wright-Giemsa morphology. B cell lineage was established by the presence of a) monoclonal sIg, as determined by indirect immunofluorescence utilizing an anti-λ or anti-κ hybridoma antibody (kindly donated by Dr. V. Raso, Sidney Farber Cancer Institute, manuscript in preparation); b) Fc and/or C3 receptors (2, 3); c) Ia antigen, by reactivity with a heterologous anti-p23,30 antiserum (8), or a monoclonal antibody of broad specificity directed against an Ia framework determinant designated I-2 (L. Nadler, manuscript in preparation). I-2 has been shown to react with B cells, adherent monocytes, and Con A-activated T cells (but not resting T cells) from 20/20 randomly selected normal individuals. In addition, I-2 reacts with homozygous typing cells encompassing all DR specificities (DR1-DR11) (cells kindly provided by Dr. Edmund Yunis, Division of Immunogenetics, SFCI). Biochemical studies (performed by Dr. John Pesando, SFCI) have demonstrated that I-2 precipitates a bimolecular complex of m.w. 29,000 and 34,000. T cell tumors were identified by spontaneous E rosette formation (>20%) and reactivity with the specific T cell antibody OKT3 (35). A total of 18 B cell tumors, including 6 chronic lymphatic leukemias (CLL), 8 diffuse, poorly differentiated lymphomas (D-PDL), and 4 nodular lymphomas were assessed for presence of B1 antigen. T cell tumors included 2 CLLs, 4 acute lymphoblastic leukemias, and 1 D-PDL. All tumor cells were cryopreserved in −196°C vapor phase nitrogen in 10% DMSO and 20% human serum until the time of surface characterization.

C-mediated lysis. C-mediated lysis was assessed as previously described (33). In brief, 5×10^6 target cells were treated with 0.2 ml ^{51}Cr sodium chromate (292 μCi/ml) (New England Nuclear, Boston, MA) and incubated for 90 min at 37°C. After 2 washes, the cells were diluted to 2×10^5/ml in media containing 10% FCS. Twenty microliters of labeled cells were distributed in conical microtiter plate wells with 20 μl of 10-fold serial dilutions of hybridoma antibody. After a 1-hr incubation at 4°C, 20 μl fresh rabbit serum (1:5 dilution), previously absorbed with 1/10 vol of normal human splenocytes (1 hr, 4°C), were added to the wells as a source of C. After an additional 1-hr incubation at 37°C, 140 μl of media were added to the wells, and the plates were spun at 400 × G for 10 min. One hundred microliters of supernatant were removed from each well and counted on a gamma scintillation counter (Packard Instrumentation Co., Downer's Grove, IL). Specific ^{51}Cr release was calculated by using the following formula:

$$\% \text{ Specific } {}^{51}\text{Cr release} = \frac{Exp - SR}{MR - SR} \times 100\%$$

where Exp = mean of the observed triplicate, SR = spontaneous release from cells incubated with C alone, and MR = maximum release obtained by treating cells with the detergent Triton X (1% solution).

Lysis of larger numbers of cells for subsequent PWM activation experiments was done by resuspending 10×10^6 mononuclear cells in 1 ml of anti-B1 antibody at a 1/100 dilution. After 1-hr incubation at 4°C, 0.25 ml fresh rabbit serum absorbed as above was added, and the cells were incubated at 37°C for an additional 60 min. Cells were then washed 3 times, resuspended in final culture medium (RPMI 1640 containing 20% FCS, 12.5 mM HEPES buffer, 4 mM L-glutamine, and 25 μg/ml gentamycin), and placed into culture.

Antibody absorption and blocking experiments. One hundred microliters of anti-B1 antibody at a 1/1000 dilution were absorbed with 10^8 viable cells from various cell populations including the immunizing Burkitt's tumor cells, E+ (T) cells, sIg+ (B) cells, monocytes, thymocytes, and the cell lines CEM (T) and SB (B). After 1-hr incubation on ice with agitation, absorbing cells were removed by centrifugation for 15 min at 400 × G, and the supernatant was harvested. Residual antibody activity was assessed by indirect immunofluorescence on a B1-positive B cell CLL target cell by methods outlined above. Forty thousand cells were analyzed on the FACS-I. The number of positive cells were enumerated by subtraction of cells staining in the presence of an unreactive monoclonal antibody, and compared with the number staining with unabsorbed anti-B1 antibody. Results were expressed as a percentage inhibition *vs* the control.

Competitive blocking experiments were conducted in order to exclude anti-B1 reactivity with an immunoglobulin determinant. One hundred microliters of anti-B1 at a dilution of 1/1000 were incubated for 1 hr at 4°C with 100 μl of a 1/2 dilution of pooled normal human serum, or 100 μl containing 500 μg of IgM or IgG myeloma protein (Cappel Laboratories, Cochranville, PA), or containing 500 μg of IgD myeloma (kindly provided by Dr. Chester Alpert, Blood Grouping Laboratory, Harvard Medical School, Boston, MA). The specificity of the myeloma protein preparations was confirmed by the selective blocking of heavy-chain specific goat anti-human Ig antisera (anti-IgM, -IgG, and -IgD) (Meloy). After incubation, 100 μl of the mixture were used for indirect immunofluorescence on a B1-positive target cell as outlined above.

Possible anti-B1 reactivity with C3 receptors was examined by attempts to inhibit EAC rosette formation with anti-B1 antibody. B cells (10^6/ml), purified by anti-Fab chromatography, were preincubated with anti-B1 (1/100 to 1/10,000) or an unreactive control ascites for 30 min at 20°C. One hundred microliters of B cells were then reacted with 100 μl of 0.5% EAC 1, 4, 2, 3 (Cordis Laboratories, Miami, FL) for 15 min at 37°C, and centrifuged at 200 × G for 10 min. The cell pellet was resuspended and 50 μl of 0.1% methylene blue were added to facilitate visualization of nucleated cells.

Fc receptor reactivity was assessed by the blocking of binding of anti-B1 to normal B cells or the immunizing Burkitt's tumor cells pretreated with heat-aggregated human IgG (see Reference 43). B cells were either untreated or incubated with aggregated IgG (10 mg/ml) for 1 hr at 20°C, washed twice, and reacted with anti-B1 at dilutions of 1/100 to 1/10,000 for indirect immunofluorescence. The number of cells staining positively in the presence and absence of aggregated IgG were enumerated on the FACS-1.

Cell cultures. Cells obtained after C-mediated lysis of the B1 population or after cell sorting were washed 3 times in tissue culture medium (TCM) and brought to 10^6/ml. One hundred microliters of cells were plated in round-bottom 96-well plates (Linbro Scientific, Hamden, CT), and 100 μl of TCM or TCM containing 50 μg/ml PWM (Difco Laboratories, Detroit, MI) were added to each well. Populations obtained by cell sorting also received 20% E+ (T) cells to facilitate B cell activation by PWM (36, 37). Plates were cultured in 95% air/5% CO_2 humid atmosphere, and cells and supernatants were harvested after 7 days for analysis of differentiation to Ig-secreting cells.

For detection of Ia antigen expression by activated T cells, 10^5 E+ cells were placed in microculture for 7 days in the presence of 5 μg/ml concanavalin A (Con A; Calbiochem, La Jolla, CA). Activated T cells, along with unstimulated control cultures and cryopreserved T cells, were harvested and prepared for immunofluorescent analysis as outlined above.

Reverse hemolytic plaque assay. Ig-secreting cells were enumerated by reverse hemolytic plaque assay as previously described (38). In brief, 50 μl of an 11% suspension of sheep erythrocytes, coated with a polyspecific rabbit anti-human Ig, together with 50 μl of lymphocytes, were pipetted into 10 x 75-mm glass tubes containing 0.9 ml of an 0.8% solution of SeaPlaque agarose (Marine Colloids, Rockland, ME), in Hanks' balanced salt solution (HBSS, Grand Island Biological Co., Grand Island, NY). Tubes were vortexed and layered over 5 ml of gelled 1.0% SeaKem agarose (Marine Colloids) in HBSS in a 60-mm Petri dish. Dishes were incubated for 1 hr at 37°C in a humid atmosphere containing 5% CO_2. One milliliter of rabbit anti-human Ig antiserum diluted 1/100 in HBSS was pipetted onto the dishes, incubated for 1 hr at 37°C, and 1 ml of guinea pig C (GIBCO) diluted 1/10 was added. Incubation was continued for an additional hour and the plaques were enumerated under a dissecting microscope.

Radioimmunoassay for Ig. The production of Ig by PWM-stimulated, fractionated cells was assessed in a solid-phase radioimmunoassay (39). Polyvinyl chloride microtiter plates (Cooke Engineering, Alexandria, VA) were coated with 100 μl of a 1/300 dilution of affinity-purified, polyvalent goat anti-human $F(ab')_2$ antibody in PBS for 2 hr at 4°C. Plates were blocked with 200 μl of 1% bovine serum albumin (BSA) in PBS for 1 hr at room temperature, and washed 3 times with PBS. Twenty-five microliters of culture medium containing various known amounts of human Ig (standards) or 25 μl of culture supernatant were added to each well, followed by 25 μl of ^{125}I-labeled human 7S Ig (Miles-Pentex, Kankakee, IL) containing approximately 8000 cpm. All culture supernatants or standards were tested in triplicate. Plates were gently agitated, covered, and incubated for 16 hr at 4°C. The contents of each well were then aspirated, the plates were washed 5 times in PBS, air-dried, and the wells were excised and counted in a gamma counter (Packard).

Results were calculated by reference to a standard curve generated at the time of each assay. Inhibition was found to be linear over a range of from 3 ng to 200 ng human Ig/25 μl.

RESULTS

Distribution of B1 antigen on normal tissues. For somatic cell hybridizations, BALB/c mice were immunized with tumor cells obtained from a patient with Burkitt's lymphoma. These cells were of B lymphocyte origin as shown by their surface phenotype: IgMκ+, Ia+, Fc+, C3+, E−, OKT3− (anti-T cell) (35), and OKM1− (anti-monocyte) (33).

A monoclonal antibody generated from this fusion, and designated anti-B1, was found, in preliminary screening on the FACS, to react strongly with the immunizing tumor (Fig. 1*A*), and was also present on 9.1 ± 2.0% of the Ficoll-Hypaque fraction of PBM from 13 normal individuals (range: 5.5 to 11.5%). In order to determine the population of cells in normal peripheral blood that bore B1 antigen, mononuclear cells from 7 individuals were separated into T, B, null, and monocyte fractions, stained with anti-B1 and G/M FITC, and analyzed on the FACS. A representative experiment is depicted in Figure 1. As shown, B1 antigen expression was limited to B cells (Fig. 1*B*), but was absent from T cells (Fig. 1*C*), monocytes (Fig. 1*D*), and null cells (not shown, but comparable to Fig. 1*D*). Staining intensity was constant at dilutions of anti-B1 ranging up to 1/2000, diminishing to background levels at 1/50,000.

A quantitative examination of peripheral blood B cells indicated that the number of Ig-positive cells in a B cell preparation

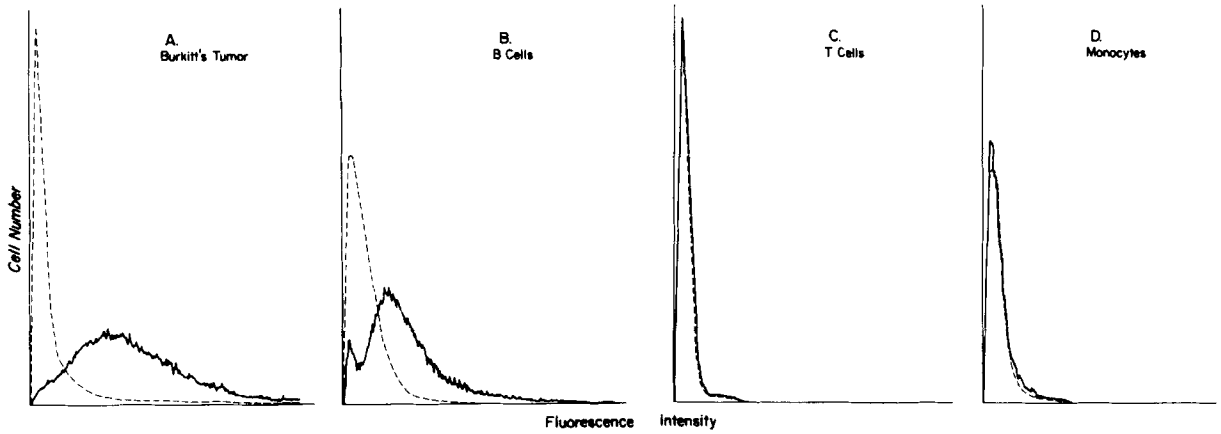

Figure 1. Reactivity of anti-B1 monoclonal antibody on separated peripheral blood mononuclear cells by indirect immunofluorescence: FACS profile. *Broken line*, control ascites, *solid line*, anti-B1. *A*, immunizing Burkitt's lymphoma; *B*, B cells; *C*, T cells; and *D*, monocytes.

was identical with the number of cells staining with anti-B1. In this experiment, cytophilic serum IgG was first removed from anti-F(ab')$_2$ immunoadsorbent-purified B cells by a 1-hr incubation of cells at 37°C (32). B cells were then stained with B1, or a mixed monoclonal anti-κ/anti-λ reagent and G/M FITC, and the number of fluorescent cells was quantitated on the FACS. Approximately 35,000/40,000 cells stained with anti-κ/λ, whereas 34,800/40,000 stained with anti-B1, thus indicating that virtually all B cells in peripheral blood are B1 positive.

A number of normal tissues were also tested for the presence of B1-positive cells. As shown in Figure 2, B1 was found on lymphocytes from tonsil (64%, n = 3), lymph node (36%, n = 5), spleen (35%, n = 3), and a small population of normal bone marrow cells (<5%, n = 3), but was undetectable on thymocytes (n = 3). The intensity of staining with anti-B1 on these other lymphoid tissues was of approximately the same magnitude as found on peripheral blood B lymphocytes.

Reactivity of anti-B1 on cell lines and tumors. The reactivity of anti-B1 was examined on a number of cell lines. All B cell lines (6/6) tested were positive for B1, as were 3/3 Burkitt's lines. Two T cell lines and a line derived from "null" cell ALL (Laz 221) were B1 negative.

Anti-B1 was also tested on a series of circulating tumor cells of lymphoid origin. All B cell tumors tested (18/18) were found to be positive for B1, whereas T cell (0/7) and myeloid tumors (0/2) were B1 negative. B1 thus appears to be limited in expression to malignancies of B cell derivation, and taken together with findings on normal tissues and cell lines, these data strongly suggest that B1 defines an antigen present only on B cells.

Cytotoxicity and absorption studies. C-mediated cytotoxicity and quantitative absorption studies were subsequently undertaken to determine if the specificity of anti-B1 was identical to that found by indirect immunofluorescence. As shown in Table I, B1 was lytic for peripheral blood B cells, but not T cells or monocytes, at dilutions of 1/100 to 1/10,000. Approximately 95% specific lysis was produced on the B cell population, which routinely contained some (5%) contamination by monocytes. Similarly, the B cell lines Laz 156 and Daudi were significantly lysed by exposure to anti-B1 and C at dilutions comparable to those effective on normal cells, whereas no lysis was produced on the T cell lines HSB2 and CEM.

A quantitative absorption experiment was also carried out. Anti-B1 at a dilution of 1/1000 was absorbed with a variety of cells of lymphoid origin, at a final concentration of 10^9 cells/ml antibody. Antibody activity remaining after absorption was assessed by binding to a B cell CLL target cell that had

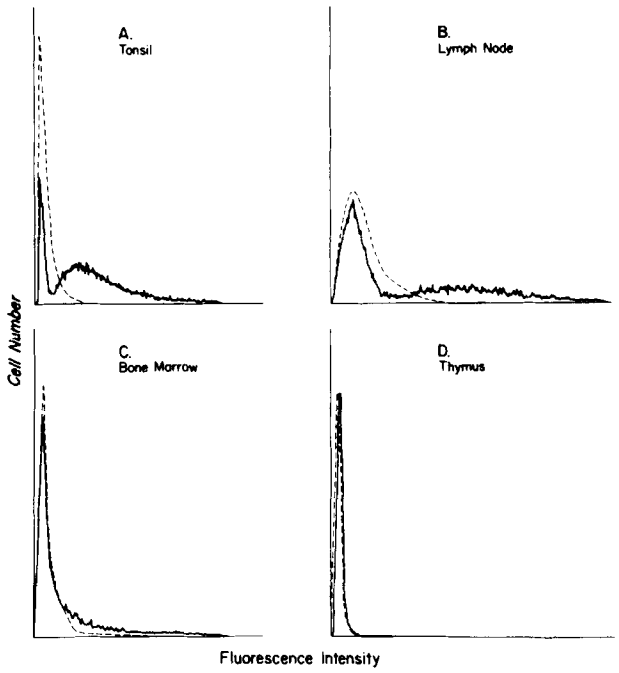

Figure 2. Reactivity of anti-B1 with normal lymphoid tissues. *Broken line*, control ascites; *solid line*, anti-B1. *A*, tonsil; *B*, lymph node; *C*, bone marrow; and *D*, thymus.

previously been found to bear B1. As shown in Table II, B1 activity was significantly removed (>93%) only by B cells, the immunizing tumor, and a B cell line (SB), but not by normal T cells, thymocytes, a T cell line (CEM), or by monocytes (<15%). The small diminution of B1 activity by absorption with the latter cells most probably reflects nonspecific adsorption of antibody, since a similar decrease in activity (approximately 10%) was noted after absorption with mouse spleen cells.

Relationship of B1 to known B cell markers. Blocking experiments were conducted in order to explore the possibility that B1 was an Ig determinant. Anti-B1 antibody at a limiting dilution of 1/2000 was preincubated with an equal volume of pooled human serum (10%) or with several human myeloma protein-rich preparations (5 mg/ml). These mixtures were then added to the appropriate B1-positive target cells (2 B1 positive lymphomas), and the intensity of staining was compared with staining produced by anti-B1 in the absence of inhibitor. Myelomas tested included those of the IgG, IgM, and IgD isotypes. Results are expressed as percent inhibition of cells staining

TABLE I

Cytotoxicity of anti-B1 on cell lines and fractionated lymphocytes

Target Cell	% ^{51}Cr Release at Anti-B1 Dilution:			
	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Laz 156	61.4 ± 3.0	56.0 ± 5.4	54.4 ± 7.2	12.7 ± 5.9
Daudi	68.5 ± 7.2	65.4 ± 2.3	62.4 ± 5.7	34.7 ± 3.0
HSB2	1.3 ± 1.7	0	0.8 ± 1.5	0.9 ± 2.6
CEM	1.0 ± 7.4	2.4 ± 7.4	0	0
Normal B cells	92.2 ± 4.7	99.5 ± 4.2	98.1 ± 4.2	13.7 ± 3.8
T cells	0	0	5.1 ± 6.1	0
Monocytes	5.9 ± 0.9	4.5 ± 0.5	5.7 ± 1.2	2.9 ± 2.1

TABLE II

Quantitative absorption of Anti-B1 antibody

Antibody Treatment	% Absorption[a]
T cells	14.7
B cells	94.5
Monocytes	12.6
CEM	13.8
SB	95.5
Thymus	10.2
Immunizing Burkitt's lymphoma	93.9

[a] % Absorption

$$= 100 - \frac{\text{No. of cells staining above bkgd, absorbed antibody}}{\text{No. of cells staining above bkgd, unabsorbed antibody}}$$

TABLE III

Failure to block B1 reactivity with myeloma proteins or serum immunoglobulin

Antibody	% Inhibition Produced by Preincubation with:			
	Pooled human serum	IgM[a]	IgG[a]	IgD[a]
B1				
Expt. 1[b]	12.3	18.6	0	0
Expt. 2[c]	5.4	2.0	9.1	9.0
Anti-IgM[b]	nd[d]	98.7	0	nd
Anti-IgG[e]	81.9	0	54.5	nd
Anti-IgD[e]	nd	5.9	0	81.0

[a] Final concentration: 2.5 mg/ml.
[b] Tested on immunizing Burkitt's lymphoma.
[c] Tested on B cell D-PDL.
[d] Not determined.
[e] Tested on tonsil.

above background in the presence *vs* absence of inhibitor. The data, presented in Table III, demonstrate that, in several experiments, no Ig-containing serum was capable of significantly inhibiting B1 reactivity. In control experiments, the reactivity of the anti-IgM, anti-IgG, and anti-IgD antisera were reduced to background levels by the corresponding myeloma-rich serum or by pooled normal human serum (Table III). Anti-IgG and anti-IgD reagents were tested on normal tonsillar lymphocytes and anti-IgM was assessed on the immunizing tumor.

Further confirmation that B1 was not an Ig determinant was demonstrated by passage of B1 antibody at a 1/2000 dilution over a human 7S γ-globulin-Sepharose immunoabsorbent column. No decrease in titer was noted by indirect immunofluorescence analysis (data not shown). Furthermore, several B cell-derived malignancies that possessed various combinations of monoclonal surface Ig, including those bearing IgMκ, IgGκ, IgMλ, and IgGλ, were all positive for B1, suggesting that B1 does not recognize an isotype-specific determinant.

The possibility that B1 represents a DR-locus specificity was also considered. Screening studies outlined above showed that adherence-purified monocytes, which are overwhelmingly Ia+ in human blood (13), were completely B1 negative (Fig. 1, Tables I and II), while at the same time reacting strongly with a monoclonal anti-Ia (p29,34) antibody. In addition, indirect immunofluorescent staining of B cells with saturating concentrations (1/100) of anti-B1 and anti-Ia, alone and in combination, always produced additive staining intensity (data not shown). At minimum this result indicates that anti-B1 and anti-Ia define different antigenic determinants. However, since T cells have also been shown to express Ia antigen subsequent to activation by antigen or mitogens (17, 18), experiments were also conducted to examine a possible relationship between Ia and B1 at the T cell level. E-rosette-purified T cells from 5 normal individuals were cultured with and without Con A for 7 days. They were then tested by indirect immunofluorescence on the FACS for the appearance of Ia and/or B1 antigen. As shown in Figure 3, T cells cultured in the presence of media alone were negative for both Ia and B1 (Fig. 3*A*). As anticipated, when T cells were exposed to Con A, approximately 25% developed Ia positivity, indicating their activated state (Fig. 3*B*). However, under these conditions, B1 antigen remained unexpressed (Fig. 3*C*). This experiment therefore provides an additional example of dissociation of Ia and B1 antigen.

Several tumors were also identified that bore large amounts of Ia antigen but were completely B1 negative. These included 2/2 patients with acute myelogenous leukemia and 3/4 patients with non-T non-B (Null cell) acute lymphatic leukemia. Thus, although B1 may represent an Ia antigen that is specific for B cells, it is clearly not present on the same molecule as detected by an anti-Ia antibody directed against an Ia framework determinant.

Finally, studies were conducted in order to demonstrate possible C3 or Fc receptor reactivity with anti-B1. Pretreatment of B cells with varying dilutions of anti-B1 failed to inhibit EAC rosette formation compared with B cells pretreated with an unreactive control ascites (54% EAC and 51% EAC, respectively). Similarly, attempts were made to block anti-B1 reactivity by pretreatment of normal B or the immunizing tumor cells with heat-aggregated human IgG. No diminution in the number of cells staining positively with anti-B1 was noted upon FACS analysis compared with B cells not exposed to aggregated IgG (31,300/40,000 *vs* 30,700/40,000 cells stained).

Determination of the proportion of B cells bearing B1: functional studies. Previous studies have indicated that PWM polyclonally induces the differentiation of B lymphocytes into Ig-secreting plasma cells (40). In order to determine whether all B cells capable of stimulation by PWM also bear B1, PBM

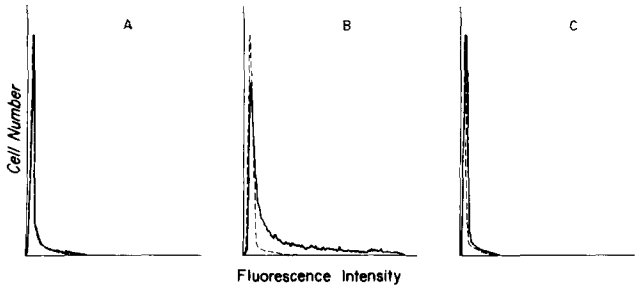

Figure 3. Lack of expression of B1 on activated T cells. *Broken line*, control ascites. *A*, T cells cultured with media; *solid line*, anti-Ia. *B*, T cells cultured with Con A (5 μg/ml); *solid line*, anti-Ia. *C*, T cells cultured with Con A; *solid line*, anti-B1.

were stained with B1 by indirect immunofluorescence and separated into B1-positive and B1-negative fractions by cell sorting. Sorted cells, along with the original, untreated population and a control population, which was stained with anti-B1 and G/M FITC but not sorted, were cultured with and without PWM. Reanalysis of the B1-negative population on the FACS confirmed that all cells staining with B1 or anti-κ/λ had been removed. Because peripheral blood B cell activation by PWM requires T cells (37, 40), 20% E-rosette positive cells were added to all cultures. At the end of 7 days, Ig production was evaluated in a radioimmunoassay (RIA) specific for human Ig. The results, summarized in Table IV, show that Ig was secreted only by unfractionated PBM and by the B1-positive lymphocyte fraction, but not by B1 negative cells, T cells, or monocytes. Ig production in all cases was dependent upon the presence of PWM in the cultures. It was observed that B1-positive cells, which comprised 8% of the unfractionated sample, did not produce proportionately greater amounts of Ig compared with the untreated control. This finding may be the result of inhibition of the response by treatment of the cells with B1 and G/M FITC, since PBM that were stained but not sorted produced a lower response than untreated PBM (220 ng/culture *vs* 950 ng/culture). Two additional experiments produced identical results.

In another approach, unfractionated PBM were treated with anti-B1 and C to determine if any B cells lacked B1 antigen and could therefore escape lysis and go on to differentiate into Ig-secreting cells after PWM stimulation. Control populations were untreated, treated with C alone, or exposed to an unreactive hybridoma antibody and C. A positive control was provided by treatment with the anti-Ia monoclonal antibody and C. All populations were then cultured for 7 days in the presence or absence of PWM. The generation of Ig-secreting cells was assessed both by direct enumeration in a reverse hemolytic plaque assay, and by analysis of culture supernatants by RIA. Three such experiments were carried out and a representative result is presented in Table V. As shown, a significant reverse plaque-forming cell (PFC) response was produced by PWM-stimulated untreated and control populations, all of which gave approximately 5000 PFC/10^6 cultured cells. In contrast, both anti-B1 and anti-Ia treatment totally abrogated the PWM-induced PFC response. Very few PFC were generated in the absence of PWM. Parallel results were obtained when supernatants from these cultures were tested for Ig content by RIA (Table V); once again, both anti-B1 and anti-Ia treated cells failed to produce Ig.

Thus, based on the ability to eliminate totally Ig secretion and reverse PFC, it appears that B1 defines the overwhelming proportion of B lymphocytes capable of PWM-induced differentiation to Ig-secreting cells.

TABLE IV

Pokeweed mitogen activation of B1+ and B1− PBM separated by cell sorting

Cell Population	ng Ig/Culture	
	−PWM	+PWM
PBL[a] (not sorted)	2.1 ± 1	950 ± 30
PBL[a] + anti-B1 (not sorted)	0	220 ± 20
B1+[a]	0	750 ± 150
B1−[a]	0	0
T cells	0	10 ± 15
Monocytes	0	1 ± 15

[a] Twenty percent T cells added.

TABLE V

Pokeweed mitogen-activation of anti-B1-treated PBM

Cell Treatment	PFC/10^6 Cultured Cells		ng Ig/Culture	
	−PWM	+PWM	−PWM	+PWM
None	120 ± 0	5920 ± 800	23 ± 5	510 ± 55
C control	220 ± 120	4620 ± 460	15 ± 1	430 ± 113
Normal ascites + C	140 ± 110	4850 ± 71	19 ± 7	680 ± 98
Anti-B1 + C	90 ± 42	40 ± 28	5 ± 1	0
Anti-Ia + C	30 ± 42	30 ± 14	0	0

DISCUSSION

In the present investigation, we describe a unique human antigen limited in expression to cells of the B lymphocyte compartment. Antigen B1, defined by the use of a monoclonal antibody, was found on approximately 9% of the PBM fraction, >95% of B cells from blood and lymphoid organs, and all tumors and cell lines of B cell lineage. Monocytes, resting and activated T cells, null cells, and tumors of T cell and myeloid derivation were uniformly B1 negative. B1 appeared to be distinct from known phenotypic markers of B cells, including surface Ig and known Ia-like antigens. Of importance, functional studies demonstrated that all cells in human peripheral blood, which can be triggered by PWM to differentiate into Ig-secreting cells, bear B1 antigen.

PWM has been widely employed as a polyclonal B cell activator, which induces the terminal differentiation of B cells to Ig-secreting plasma cells (40). Elimination of the B1+ population with anti-B1 and C or by cell sorting abrogated PWM-stimulated Ig secretion, as determined both by a RIA and a reverse hemolytic plaque technique for detecting individual Ig-secreting cells. Both methods employed a polyvalent anti-Ig antiserum that detects all human Ig isotypes. Taken together with the results from immunofluorescence and cytotoxicity studies that >95% of Ig+ cells are also B1+, it would seem that few B1−B cells could be present. Nevertheless, it remains possible that a small B cell subpopulation, which is both B1− and unstimulatable by PWM, does exist in human peripheral blood. B cell heterogeneity has been observed in the mouse with respect to PWM activation (40), although the behavior of human B cells in this regard is presently unclear. In addition, PWM-induced differentiative responses in human peripheral blood are highly T cell dependent (37, 40), more specifically helper cell dependent (41), and failure of activation may reflect suboptimal T helper influence rather than an inherent functional property of a B cell subpopulation. At the present time we feel that the weight of evidence from both functional and reactivity studies supports the interpretation that most, if not all normal, resting B cells bear B1 antigen.

For the purpose of B cell enumeration B1 possesses the advantage of defining a non-Ig determinant, thus circumventing technical problems related to the binding of serum IgG to cellular Fc receptors (14). Our finding that 9.1% of the peripheral blood mononuclear fraction reacts with anti-B1 is in close agreement with values obtained for integral membrane Ig+ cells obtained after temperature (32) or pH-induced (42) dissociation of cytophilically-bound IgG (8.9% and 9.7%, respectively), or as detected by pepsin-digested anti-Ig reagents (6.3%) (14). B1 should therefore prove to be extremely useful in the isolation and functional analysis of pure B cell populations, as well as in the identification and enumeration of B cells in congenital and acquired immunodeficiency disorders, and in malignancies of B cell derivation.

B1 appears to be distinct from classical B cell phenotypic

markers, particularly Ia antigen and Ig determinants. Antisera with B cell specificity have been previously described, which are in fact directed against Ia-like antigens encoded by the HL-A-D locus (5–8). Such antigens are present on numerous other cell types, including nonlymphoid cells (24), in addition to B lymphocytes. In the present studies, B1 reactivity failed to coincide with that of anti-Ia, in the lack of B1 expression on monocytes and activated T cells (Fig. 1; Tables I and II), and in the identification of a number of tumors that were Ia+, B1−. B1 reactivity could not be inhibited by a variety of human Ig-containing preparations (Table III). Finally, attempts to demonstrate possible Fc or C3 receptor reactivity of anti-B1 were unsuccessful. No inhibition of B1 reactivity was produced by pretreatment of B cells with aggregated human IgG, nor were the number of EAC rosettes in a B cell population reduced by pretreatment of cells with a range of dilutions of anti-B1.

Other B cell-specific antigens have been described, which appear, like B1, to be distinct from Ig and Ia-like antigens on the basis of tissue distribution and/or m.w. (19–23). These include heteroantisera-defined antigens that apparently identify B cell subpopulations (23, 44), as well as antigens with broad B cell representation (20, 22). Further studies are currently in progress in our laboratory to determine the molecular characteristics of B1, its functional relevance, and its possible relationship to other previously described antigens.

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A Unique Cell Surface Antigen Identifying Lymphoid Malignancies of B Cell Origin

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ABSTRACT A monoclonal antibody (anti-B1) specific for a unique B cell surface differentiation antigen was used to characterize the malignant cells from patients with leukemias or lymphomas. All tumor cells from patients with lymphomas or chronic lymphocytic leukemias, bearing either monoclonal κ or λ light chain, expressed the B1 antigen. In contrast, tumor cells from T cell leukemias and lymphomas or acute myeloblastic leukemias were unreactive. Approximately 50% of acute lymphoblastic leukemias (ALL) of non-T origin and 50% of chronic myelocytic leukemia in blast crisis were also anti-B1 reactive. Moreover, 21 of 28 patients with the common ALL antigen (CALLA) positive form of ALL were anti-B1 positive, whereas 0 of 13 patients with CALLA negative ALL were reactive.

These observations demonstrate that an antigen present on normal B cells is expressed on the vast majority of B cell lymphomas and on ~75% of CALLA positive ALL, suggesting that these tumors may share a common B cell lineage.

INTRODUCTION

Leukemias and lymphomas, which were not previously distinguishable by either morphologic or histochemical criteria, can now be subdivided into clinically and pathologically distinct subgroups by use of a number of cell surface markers expressed on normal lymphocytes (1–3). For example, both normal and malignant B cells are defined by their expression of cell surface immunoglobulin (4, 5). Other markers of the B cell membrane, including receptors for the Fc portion of human immunoglobulin (6), receptors for components of the complement system (7), and HLA-D-related Ia-like antigens (8, 9) are less useful because they are not restricted to cells of B lineage and are also found on normal and malignant monocytes (10–12). In addition, Fc receptor-bound immunoglobin may give spuriously positive results for cell surface immunoglobulin (13). Although T cells have been shown to be reactive with anti-T cell antisera (14, 15), or to form erythrocyte rosettes with sheep erythrocytes (16), they, too, may express Fc or C3 receptors or Ia-like antigens (17–19). Finally, Null cells, which lack the conventional markers of T and B cells (20), also have been shown to express C3, Fc, or Ia-like antigens (21–23). Given the extent of overlap of many of these cell surface markers, considerable attention has been directed at defining unique cell surface antigens present on normal T, B, and Null cells, which can then be used to identify and classify leukemias and lymphomas.

In a recent study (24), we described the development and characterization of a monoclonal antibody (anti-B1) that is reactive with a differentiation antigen expressed on all human B cells and on those cells destined to differentiate into immunoglobulin-secreting cells under pokeweed mitogen stimulation. The B1 antigen has been shown to be distinct from other known phenotypic markers of B cells, including surface immunoglobulin, Fc and C3 receptors, and Ia-like antigens. More importantly, anti-B1 was unreactive with normal T lymphocytes, Null cells, and granulocytes. In the present study, we have used anti-B1 to characterize a large number of malignancies thought to be of T, B, monocyte, myeloid, and Null cell origin. These studies demonstrate that anti-B1 reacts only with those B cell lymphomas that express either monoclonal κ or λ light chain. Of considerable interest is the demonstration that tumor cells from the majority of patients with the common acute lymphoblastic leukemia anti-

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gen (CALLA)[1] positive non-T cell form of acute lymphoblastic leukemia (ALL) and chronic myelocytic leukemia (CML) in blast crisis were also reactive with anti-B1 antibody. This study supports the notion that most B cell lymphomas and many CALLA$^+$ ALL share a common B cell lineage.

METHODS

Patients and sample preparations. All patients in this study were evaluated at the Sidney Farber Cancer Institute, the Children's Hospital Medical Center, Peter Bent Brigham Hospital, Beth Israel Hospital, or the Massachusetts General Hospital. The diagnosis of lymphoma or leukemia was made using standard clinical, morphologic, and cytochemical criteria (25–27). Heparinized peripheral blood or bone marrow was collected from leukemic patients or from patients with circulating lymphomas (lymphosarcoma cell leukemias) before the administration of chemotherapeutic agents or blood products. Lymphocytes were separated from these specimens by Ficoll-Hypaque density gradient centrifugation, as previously described (28). Tumor masses and lymphoid tissue from patients with lymphomas were gently teased, minced into single cell suspensions, and passed through stainless steel mesh wire filters. Tumor cells were readily distinguishable from normal lymphocytes by Wright-Giemsa morphology, and all neoplastic preparations selected for this study had >75% abnormal cells. Isolated tumor cells were studied either fresh or cryopreserved in 10% dimethyl sulfoxide and 20% fetal calf serum at −196°C in the vapor phase of liquid nitrogen until the time of surface characterization.

Preparation of normal lymphocyte subpopulations. Human peripheral blood mononuclear cells were isolated from normal volunteer adult donors by Ficoll-Hypaque density gradient centrifugation. Normal lymphoid tissues from tonsil, lymph node, spleen, and thymus were prepared as described above. Unfractionated cells were then separated into B cell (surface immunoglobulin [sIg] positive), T cell (sheep erythrocyte rosette [E] positive), monocyte (adherent), and Null cell (sIg$^-$, E$^-$) by standard techniques (29). In particular, the B cell preparations were routinely >90% sIg$^+$ and <5% E$^+$, nonreactive with anti-T cell antibodies, and ~5% monocytes as judged by morphology, latex ingestion, and reactivity with the monocyte-reactive monoclonal antibody (M1) (30). The T cell populations obtained were <2% sIg$^+$ and >95% E$^+$, uniformly reactive with anti-T cell antibodies, and entirely negative with M1. Normal monocytes were obtained by adherence to plastic dishes as previously described (30), and were 95% M1$^+$, but did not form erythrocyte rosettes, react with anti-T cell antisera, or express sIg. Null cells were sIg$^-$, E$^-$, and T cell antisera negative.

Cell surface markers. The cellular lineage of tumor cells was determined by a number of cell surface markers. The definition of T cell lineage was established by reactivity with a T cell-specific heteroantiserum (14) and monoclonal antibodies (15), and by reactivity with sheep erythrocytes as previously described (16). All the T cell leukemias and lymphomas were >75% reactive with the T cell-specific monoclonal antibodies and heteroantiserum, and these tumor cells were uniformly >20% erythrocyte rosette reactive (31, 32).

The B cell derivation of the tumor cell was demonstrated by the expression of either monoclonal κ or λ light chains on the tumor cell surface. Monoclonal antibodies specific for κ or λ light chain were used in all studies (provided by Dr. Victor Raso, Sidney Farber Cancer Institute, Boston, Mass.). In addition, a monoclonal antibody specific for the framework of the human HLA-D-related Ia-like antigen was used to analyze all normal and malignant cells for reactivity. The Ia-like antigens are gene products of the HLA-D region, which are present on the surface of normal peripheral blood B cells, a fraction of Null cells, monocytes, and activated T cells, but not on resting T cells (33). These Ia-like antigens have not been detected on the vast majority of T cell leukemias and lymphomas, but are expressed on most hematopoietic non-T cell malignancies. The anti-Ia antibody used in this study appears to be identical to the previously described heteroantiserum (19) and monoclonal antibodies (33), which identified a common framework expressed on all Ia-like antigens.

The non-T cell leukemias were characterized using a monoclonal antibody (J-5) (34), which has been shown to have the specificity of a previously described rabbit anti-CALLA antisera prepared in this laboratory (35).

The preparation and characterization of the anti-B1 antibody was the subject of a previous report (24). In brief, anti-B1 was developed by somatic cell hybridization, was cloned by limiting dilution, and has been passaged in ascites form in BALB/c mice for over 1 yr. Ascites form anti-B1 was used for all experiments. This antibody has been shown to be of the IgG_2 subclass and can induce lysis of reactive cells with rabbit complement at dilutions up to 1:50,000. By indirect immunofluorescence, cytotoxicity, and quantitative absorption, the B1 antigen was present on >95% of B cells from blood and lymphoid organs in all individuals tested. Monocytes, resting and activated T cells, Null cells, myeloid cells, and T cell lines were B1 antigen negative. The B1 antigen was shown to be distinct from human immunoglobulin isotypes, Ia-like antigens, Fc receptor of immunoglobulin, and the C3 receptor. Functional studies demonstrated that removal of the B1 antigen positive population from peripheral blood by cell sorting or complement-mediated lysis eliminated the cell population that is induced to differentiate into immunoglobulin-secreting plasma cells by pokeweed mitogen.

Indirect immunofluorescence analysis of normal and malignant cells with monoclonal antibodies. Normal or malignant cells were used fresh or thawed and washed extensively at the time of study; their viability exceeded 85% in all cases. In brief, 1–2 × 10^6 cells were treated with either 0.1 ml of a 1:500 dilution of the specific monoclonal antibody to be tested or 0.1 ml of a 1:500 dilution of an unreactive control antibody of a similar immunoglobulin isotype, incubated at 4°C for 30 min, and washed three times. These cells were reacted with 0.1 ml of a 1:40 dilution of fluorescein-conjugated goat anti-mouse IgG (G/M FITC) (Meloy Laboratories, Inc., Springfield, Va.), incubated at 4°C for 30 min, washed three times, and analyzed as previously described (36). Intensity of fluorescence was determined for 40,000 cells in each population on a fluorescence-activated cell sorter and compared with the fluorescence of a control nonreactive ascites. A displacement of the histogram of the test monoclonal antibody (Fig. 2A) was scored positive compared with the histogram of an unreactive isotype identical monoclonal antibody. In addition, for each test sample, a quantitative assessment of the number of positive cells was made (number of cells reactive with test monoclonal antibody minus number of cells reactive with the unreactive isotype identical monoclonal antibody divided by 40,000 total cells tested). Because the fluores-

[1] *Abbreviations used in this paper*: ALL, acute lymphoblastic leukemia; CALLA, common acute lymphoblastic leukemia antigen; CLL, chronic lymphocytic leukemia; CML, chronic myelocytic leukemia; E, sheep erythrocyte rosette; G/M FITC, fluorescein-conjugated goat anti-mouse IgG; sIg, surface immunoglobulin.

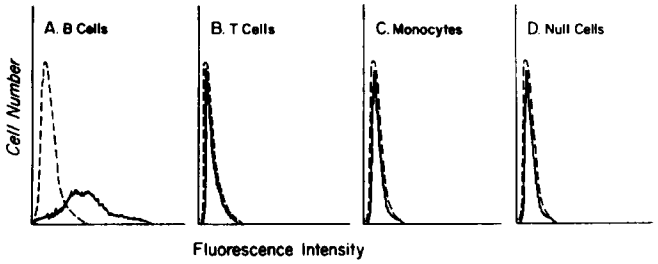

FIGURE 1 The fluorescence profile of fractionated B cells (A), T cells (B), monocytes (C), and Null cells (D) with anti-B1 antibody and G/M FITC (solid line) is depicted in this figure. It is seen that B cells react with the anti-B1 antibody. Background fluorescence staining (dotted line) was obtained by incubating cells with an unreactive monoclonal antibody and developing with G/M FITC.

cence intensity of these antibodies was not different in dilutions from 1:50 to 1:10,000 or greater, it appeared that the intensity of reactivity related to the number of specific antigen-reactive determinants on the cell surface.

RESULTS

Distribution of B1 antigen on normal hematopoietic tissues. As was previously shown (24), anti-B1 identified a surface antigen present on ~9% of unfractionated peripheral blood mononuclear cells. Mononuclear cells from several individuals were separated into T, B, Null, and monocyte fractions and analyzed for reactivity using anti-B1 and G/M FITC. As shown in Fig. 1, the B1 antigen was found uniquely on B cells (Fig. 1A), and was absent from T cells (Fig. 1B), monocytes (Fig. 1C), and Null cells (Fig. 1D). Moreover, the B1 antigen was present on the Ig^+ cells from tonsil (64%; $n = 3$), lymph node (36%; $n = 12$), spleen (35%; $n = 8$), and a small population of normal bone marrow (5%; $n = 5$), but was not detected on thymocytes ($n = 4$). The intensity or amount of reactivity of these tissues with anti-B1 was similar to that found on peripheral blood B cells.

Reactivity of anti-B1 with malignant lymphomas. Anti-B1 was tested for reactivity with the tumor cells from patients with B cell lymphomas. These tumors were determined to be of B cell origin by the presence of cell surface monoclonal κ or λ light chains and by their failure to form erythrocyte rosettes or react with anti-T cell antisera. The tumors were classified according to the scheme of Rappaport (37) and included the following histologic types: (*a*) diffuse, poorly differentiated lymphocytic ($n = 18$); (*b*) diffuse histiocytic ($n = 7$); (*c*) nodular, poorly differentiated lymphocytic ($n = 8$); (*d*) Burkitt's lymphoma ($n = 9$); (*e*) nodular mixed ($n = 3$); (*f*) Waldenstrom's ($n = 2$); and (*g*) myeloma ($n = 3$). In contrast to the patterns obtained with normal B cells, the reactivity of anti-B1 with these B cell tumors varied, and is illustrated in Fig. 2. For example, tumor cells from patients with chronic lymphocytic leukemia (CLL) were weakly but definitively reactive with anti-B1 (Fig. 2A). In contrast, the tumor cells from patients with nodular, poorly differentiated lymphocytic tumors (Fig. 2B) were moderately reactive, whereas the tumor cells from patients with Burkitt's lymphoma were strongly reactive (Fig. 2C). Of considerable interest was the finding that all plasma cell myelomas tested were unreactive (Fig. 2D). The results obtained with cells of 50 patients with B cell lymphoma are summarized in Table I. The tumor cells from all 47 patients with classical B cell lymphoma were reactive with anti-B1, anti-Ia, and either anti-κ or anti-λ light chain, but not both. The three plasma cell myelomas tested were unreactive with anti-B1. The tumor cells from two of these patients lacked both the Ia antigen and surface κ or λ; however, the tumor cells from the third patient expressed both. In contrast, only one of three patients with Null cell lymphoma was $B1^+$, although the tumor cells from all three of these patients were reactive with an anti-Ia antisera. Moreover, all 13

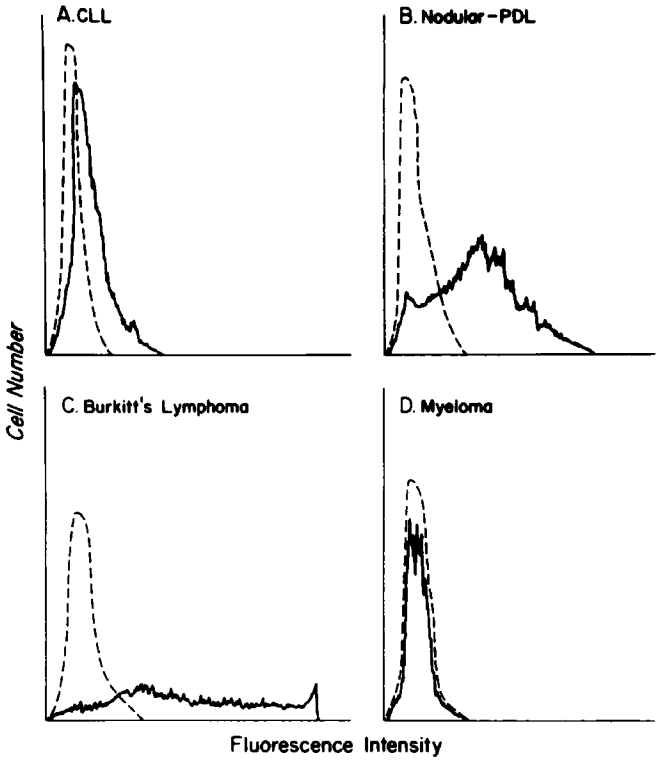

FIGURE 2 The fluorescence profile of tumor cells from patients with B cell CLL (A), nodular, poorly differentiated lymphocytic lymphoma (B), Burkitt's lymphoma (C), and plasma cell myeloma (D) with anti-B1 antibody and G/M FITC (solid line) is depicted in this figure. It can be seen that tumor cells from patients with CLL are weakly reactive, nodular, poorly differentiated lymphocytic cells are moderately reactive, Burkitt's lymphoma strongly reactive, and plasma cell myelomas are unreactive with anti-B1. Background fluorescence staining was performed as in Fig. 1.

TABLE I
Reactivity of Lymphomas and Leukemias with Anti-B1

Tumor	Number of patients	Number reactive with antisera			
		Ia	B1	κ or λ	Anti-T cell
Lymphomas					
B cell	50	48	47	48	0
Null cell	3	3	1	0	0
T cell	13	0	0	0	13
Leukemias					
CLL	18	18	18	18	0
ALL—non-T	41	41	21	0	0
ALL—T cell	17	0	0	0	17
CML—stable phase	6	4	0	0	0
CML—blast crisis	10	7	5	0	0
AML*	16	15	0	0	0

* AML, acute myeloblastic leukemia.

T cell lymphomas tested were unreactive with anti-Ia, anti-B1 and anti-κ or anti-λ monoclonal antibodies.

Reactivity of anti-B1 with leukemias. Since the vast majority of sIg-bearing (B cell) lymphomas were reactive with anti-B1, we next evaluated the expression of the B1 antigen on leukemic cells. As can be seen in Table I, the tumor cells from patients with CLL expressed monoclonal surface κ or λ light chain, Ia antigen, and B1 antigen. An unexpected result was noted when the ALL cells were tested with anti-B1. It was found that the tumor cells from ~50% of the patients with non-T cell ALL were $B1^+$. Leukemic cells from these individuals lacked surface κ or λ, but were $B1^+$ and Ia^+. T cell ALL were unreactive with anti-B1 and anti-Ia. In contrast, tumor cells from patients with acute myeloblastic leukemia were generally Ia^+ and uniformly lacked the B1 antigen. These findings provided additional support for the view that the anti-B1 was unreactive with conventional sIg or Ia-like antigens. In addition, it was found that 5 of 10 patients with CML in blast crisis were $B1^+$, whereas 0 of 6 patients with stable phase CML were unreactive with anti-B1.

Coexpression of B1 antigen and CALLA on non-T cell leukemias. Given the reactivity of anti-B1 with some, but not all, non-T cell ALL, the relationship of the B1 antigen to CALLA was then investigated. Previous studies have shown that CALLA is a tumor-associated antigen expressed on the leukemic cells from 80% of

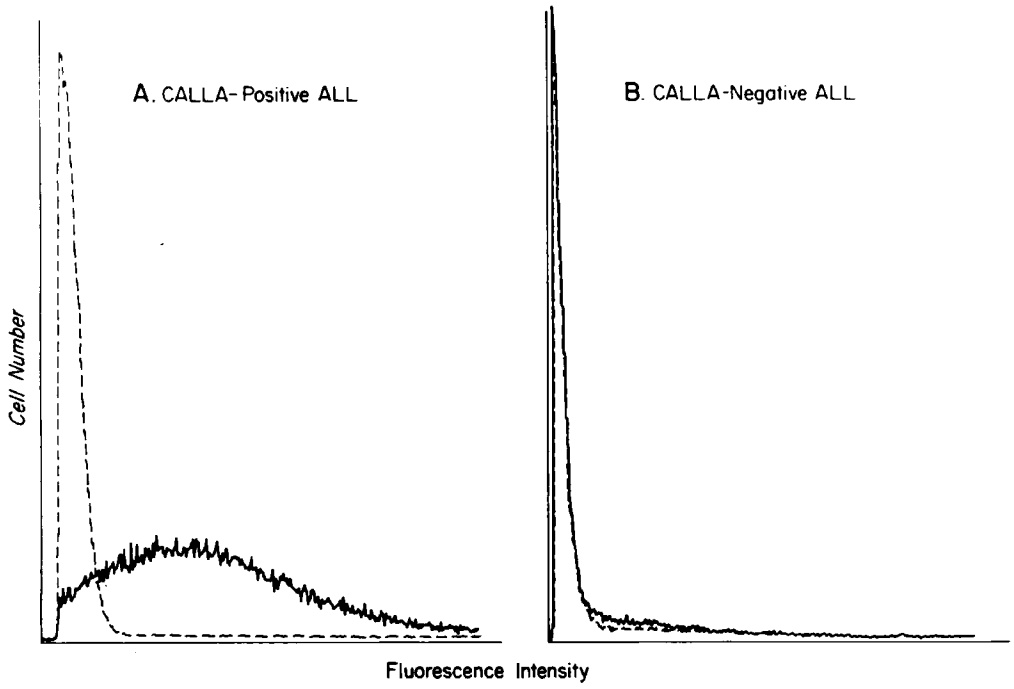

FIGURE 3 The fluorescence profile of the tumor cells from a patient with a $CALLA^+$ ALL (A) and $CALLA^-$ ALL (B) with anti-B1 antibody and G/M FITC (solid line) is depicted in this figure. It can be seen that the tumor cells from the $CALLA^+$ ALL were uniformly reactive with anti-B1 (A), whereas the tumor cells from the $CALLA^-$ patient were unreactive (B). Background fluorescence staining was performed as in Fig. 1. The tumor cells from approximately one-third of patients with $CALLA^+$ ALL had an identical fluorescence pattern to the one depicted in Fig. 3A. The fluorescence intensity patterns of the two-thirds of patients with $CALLA^+$ ALL were equally divided between weak expression (similar to Fig. 2A) and moderate expression (similar to Fig. 2B).

TABLE II
Reactivity of Anti-B1 with CALLA Positive and Negative Leukemic Cells

Tumor	Number of patients	Number reactive with antisera	
		Ia	B1
Non-T cell ALL, CALLA$^+$	28	28	21
Non-T cell ALL, CALLA$^-$	13	13	0
CML—blast crisis, CALLA$^+$	7	6	5
CML—blast crisis, CALLA$^-$	3	1	0

patients with non-T cell ALL and ~30% of patients with CML in blast crisis (38). This antigen has been shown to be a glycoprotein with a molecular mass of 100,000 daltons. Recently, a monoclonal antibody (J-5), specific for CALLA, has been described. The tumor cells from 41 patients with non-T ALL and 10 patients with CML in blast crisis were then compared for their reactivity with anti-Ia, anti-CALLA, and anti-B1 monoclonal antibodies. The reactivity of anti-B1 with the tumor cells from a CALLA positive and a CALLA negative patient is depicted in Fig. 3. Fig. 3A shows that the tumor cells from a patient with CALLA$^+$ ALL were reactive with the anti-B1 antibody, whereas Fig. 3B shows that the tumor cells from a patient with CALLA$^-$ ALL were unreactive. Further heterogeneity of the CALLA$^+$ ALL and CML in blast crisis could be demonstrated by their reactivity with anti-B1. As shown in Table II, the tumor cells from 21 of 28 patients with CALLA$^+$, Ia$^+$ ALL were reactive with anti-B1. In contrast, no tumor cells from the 13 patients with CALLA$^-$, Ia$^+$ ALL were reactive. Similarly, most of the CALLA$^+$, Ia$^+$ CML in blast crisis were anti-B1 reactive; and all of the CALLA$^-$, Ia$^+$ CML in blast crisis were unreactive.

DISCUSSION

In the present study, we have used a monoclonal antibody previously shown to be specific for a B cell surface-differentiation antigen to characterize malignant cells from patients with leukemias and lymphomas of various cellular origins. Examinations of the non-Hodgkin's lymphomas with classical cell surface markers demonstrated that ~80% of these tumors and >95% of CLL are of B cell lineage (3). Morphologically, the B cell lymphomas are heterogeneous, and the observed histologic diversity has led to the development of several classification schemes (37–40). These B cell tumors have also been shown to be variable in their amount of expression of surface or intracytoplasmic immunoglobulin, complement receptors, formation of monkey erythrocyte rosettes, Fc receptors, and Ia-like antigens. It has therefore been postulated that the cell surface marker and histologic diversity seen in these tumors may reflect distinct stages of B cell differentiation in which the malignant cells are "frozen" (41). Unfortunately, the various cell surface markers presently used define neither unique histologic subtypes nor distinct clinical subgroups. Nevertheless, given the better prognosis of B cell neoplasms compared with T or Null cell tumors, a number of these markers have been widely used (42, 43).

In this study, the tumor cells from all 18 patients with B cell CLL and 47 of 50 patients with B lymphomas, all bearing κ or λ light chains, were reactive with the anti-B1 antibody. Moreover, anti-B1 was unreactive with acute T cell leukemias and lymphomas and with tumor cells from all patients with acute myeloblastic leukemia. These observations suggest that anti-B1 adds to the repertoire of cell surface determinants that define B cell tumors, and unlike Ia, Fc, and C3, it is restricted to this class of cells. More importantly, the presence of B1 antigen in conjunction with the expression of monoclonal κ or λ light chains provides additional criteria for the definition of a malignant B cell clone. In this regard, normal B cells and other cells capable of binding immunoglobulin via an Fc receptor are invariably heterogeneous with regard to their light chain phenotype, and as such, can be distinguished from B cell neoplasms.

Although anti-B1 was reactive with the vast majority of B cell lymphomas and all B cell CLL, the non-T cell ALL were divided into several distinct entities. These tumor cells have been shown to be unreactive with anti-T and anti-Ig reagents, but to be strongly reactive with anti-Ia and anti-CALLA. Previous studies have shown that 95% of non-T cell ALL are Ia$^+$, whereas CALLA was coexpressed on ~80% of the non-T cell ALL. These studies indicated that the majority of non-T cell ALL were CALLA$^+$, Ia$^+$, and a small group were CALLA$^-$, Ia$^+$. Little is known about the small subset of patients (1–2%) who express cell surface immunoglobulin and are therefore thought to represent a more mature B cell ALL.

The present studies have shown that the tumor cells from ~50% of patients with non-T cell ALL were reactive with anti-B1. More importantly, most of the CALLA$^+$, Ia$^+$ ALL were anti-B1 reactive, whereas all of the CALLA$^-$, Ia$^+$ ALL were anti-B1 unreactive. Thus, the non-T cell ALL can now be divided into three major subclasses: (*a*) CALLA$^+$, Ia$^+$, B1$^+$; (*b*) CALLA$^+$, Ia$^+$, B1$^-$; and (*c*) CALLA$^-$, Ia$^+$, B1$^-$. These studies provide additional support for the view that a significant fraction of CALLA$^+$ ALL was B cell derived. Other investigators have demonstrated that ~20–30% of CALLA$^+$ ALL had the characteristics of pre-B cells in that they contained intracytoplasmic μ chain and lacked both surface and cytoplasmic light chains (44–46). The present study would suggest that, in fact, the majority

of CALLA$^+$, Ia$^+$ ALL are B cell derived, since 75% were anti-B1$^+$. Whether B1 is expressed earlier than cytoplasmic immunoglobulin in B cell differentiation, or is a more sensitive marker of pre-B cells, is yet to be resolved.

The demonstration that the B1 antigen is expressed on all normal B cells, B cell lymphomas, and a proportion of acute leukemias suggests that the B1 antigen is expressed on most stages of B cell differentiation. It was intriguing to find that the generally accepted end-stage cell in B cell ontogeny, the plasma cell, lacked B1. Thus, B1 appears to be a B cell differentiation antigen present throughout most stages of B cell maturation. Similarly, a number of anti-T cell antibodies have been described that are capable of dissecting normal intra- and extrathymic maturation (47), as well as defining distinct subsets of clinically relevant malignant T cell leukemias and lymphomas (31, 32, 38, 48, 49).

Because monoclonal antibodies are of extremely high titer and can be produced in unlimited quantities compared with heteroantisera, the utility of this marker can now be readily adopted by many laboratories studying B cell tumors. Additional B cell-specific antibodies will be required for the dissection of distinct stages of B cell differentiation and for the identification of clinically relevant subgroups of B cell lymphoproliferative malignancies.

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Production of a mouse-human chimeric monoclonal antibody to CD20 with potent Fc-dependent biologic activity.

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Mouse monoclonal antibody 2H7 recognizes the CD20 cell surface phosphoprotein that is expressed in normal as well as malignant B cells. CD20 may be a useful target for therapy of B cell lymphomas, since damaged normal B cells can be replaced by their antigen-negative precursors. Monoclonal antibody 2H7 is an IgG2b (kappa) immunoglobulin which cannot mediate antibody-dependent cellular cytotoxicity with human lymphocytes or complement-dependent cytotoxicity with human serum. We have now generated a chimeric 2H7 antibody by substituting the mouse constant domains of 2H7 with the human gamma 1 and kappa domains. This new antibody has the same binding specificities as 2H7 but is highly effective in mediating antibody-dependent cellular cytotoxicity with human effector cells and complement-dependent cytotoxicity with human complement.

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Rapid Communication

Expression of B-cell Antigens by Hodgkin's and Reed–Sternberg Cells

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Twenty frozen and 55 paraffin sections of lymph-node specimens from 55 patients with pretreatment Hodgkin's disease (nodular sclerosis Hodgkin's disease, n = 45; mixed cellularity Hodgkin's disease, n = 10) were studied by immunohistochemistry and molecular analysis to determine the phenotype of Hodgkin's and Reed–Sternberg cells (HRS). In all cases the HRS cells were CD45−, and CD30+, and in 43/55 (78%) cases they were CD15+. In 48/55 cases (87%) HRS cells were reactive with at least one B-cell marker (CD19, CD20, CD22, CDw75, MB2), 8/55 cases (14.5%) showed reactivity (mainly cytoplasmic) of a subpopulation of HRS cells with the T-cell markers CD3 and βF1. All cases that expressed T-cell antigens were also reactive with at least one B-cell marker. In frozen sections, a minority of HRS cells in each case studied showed cytoplasmic positivity for bcl-2 protein. Rearrangement of immunoglobulin heavy chain genes was detected in one case and of T-cell receptor β chain genes in none. The authors were unable to confirm previous reports of bcl-2 gene rearrangement in Hodgkin's disease. The results strongly support a B lymphocytic origin of HRS cells. (Am J Pathol 1991, 139:701–707)

Despite numerous studies on the histogenesis of Hodgkin and Reed–Sternberg (HRS) cells, their nature still remains enigmatic. Using a wide variety of approaches derivation has been suggested from macrophages,[1–6] interdigitating reticulum cells,[7–9] activated lymphocytes,[10] immature lymphoid cells,[11] B cells[12–19] and T cells.[20–25]

Immunohistochemical and molecular genetic evidence is accumulating that HRS cells are derived from either B or T lymphocytes rather than macrophages and interdigitating reticulum cells with most immunohistochemical studies favoring a T-cell derivation of these cells.[20–25] Most of these studies favoring a T-cell derivation of HRS cells have, however, used a limited panel of mono- or polyclonal antibodies specific for B and T cells and in some instances have used T-cell markers alone.[22,24]

In contrast to immunohistochemistry most molecular studies[13,14,17,26–28] have pointed towards a B-cell phenotype of HRS cells. Furthermore, the recent report of bcl-2 gene rearrangement in a significant number of Hodgkin's disease cases,[19] if confirmed, would be strong evidence in this direction.

We report the results of a combined immunohistochemical and molecular study of 55 cases of Hodgkin's disease (excluding the lymphocyte predominant subtype). We have used a broad panel of antibodies against both B and T cells performed genotypic analysis by Southern blotting and have attempted to confirm the presence of bcl-2 gene rearrangement as reported by Stetler–Stevenson et al.[19]

Material and Methods

Immunohistochemistry

Seventy-five specimens from 55 patients with pretreatment Hodgkin's disease (55 paraffin sections, 20 frozen sections) were taken from the surgical pathology files of the Department of Pathology, University College and Middlesex School of Medicine, London, and stained with

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the panel of monoclonal and polyclonal antibodies listed in Table 1. The alkaline phosphatase anti–alkaline-phosphatase (APAAP) method[29] and a modification of the ABC method[30] were used for frozen and paraffin sections, respectively. Paraffin sections were predigested with trypsin (Sigma) when using the antibodies CD15, CD30, CD3 (polyclonal), and βF1. Each specimen was classified according to the Rye classification as either nodular sclerosis Hodgkin's disease (NSHD) (n = 45) or mixed cellularity HD (MCHD) (n = 10). Nodular lymphocyte predominant Hodgkin's disease (NLPHD) was not included in this study. The percentage of cells staining with antibodies specific for B and T cells was estimated as less than 10, 10–50, and over 50. Attention was given to ensure that the immunoreactive HRS cells were unequivocally tumour cells, especially in cases where less than 10% of HRS cells expressed the respective antigens (e.g., the presence of characteristic nuclear details and surrounding T-cell rosettes). All cases were examined separately by two of the authors (CS, PGI). The distribution of staining, i.e., cytoplasmic, surface or both was also noted.

Molecular Genetics

Gene Rearrangement Analysis

High molecular weight DNA was extracted from 18 frozen biopsy specimens of HD as described previously.[31] The purified DNA was digested separately with three restriction enzymes (EcoR1, HindIII, PstI). The resulting digests were size-fractionated on 0.8% agarose gels and transferred to Hybond N-plus membranes (Amersham, UK) by Southern blotting. The recombinant DNA probes used included those encoding the J_H region of the immunoglobulin (Ig) heavy chain[32] and the constant region of the β chain of the T-cell receptor (TCRβ).[33] They were radiolabelled with 32 P-dCTP by the random hexamer method.[34] After the hybridization, the membranes were washed under stringent conditions and exposed to prefogged X-ray film at −70°C.

Polymerase Chain Reaction (PCR)

Fresh frozen tissue of 19 specimens and paraffin material from 32 cases (all of the paraffin sections showing reactivity of HRS cells with CD20) were studied using PCR.[35] Two sets of PCR were carried out for each sample: one using primers designed to amplify the major breakpoint region of t(14;18),[36] the other using primers designed to amplify a fragment of the normal β-globin gene,[35] as a control. Forty-five cycles of PCR were carried out on a thermal cycler (Hybaid, UK) using a mixture of 1 unit of Taq polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM MgCl, 0.001% gelatine, 200 μM each dNTP, 200 ng of DNA or one dewaxed paraffin section, and 250 ng of DNA primers (obtained from Oswell DNA Service, Edinburgh, UK). PCR products were visualized on an ethidium bromide stained 3% agarose gel and analyzed by conventional Southern blotting,[31] using a radiolabelled probe to the bcl-2 major breakpoint (pFL3).[37] A follicular lymphoma with known bcl-2 major breakpoint rearrangement was used as positive control. Extracted placental DNA and dewaxed sections of reactive tonsillar tissue were used as positive controls for the β-globin amplification and as negative controls for the major breakpoint amplification.

Results

Immunohistochemistry

The results obtained with antibodies specific for B- and T-cells in 20 frozen sections are listed in Table 2. In all

Table 1. *Details of Antibodies Employed and Their Source*

CD-antigen	Antibody	P/F	Source	Ig class	Major lymphoid specificity
CD19	HD37	F	1	Ig G1	B cells
CD20	L26	P/F	1	Ig G2a	B cells
CD22	4KB128	F	2	Ig G1	B cells
CDw75	LN1	P	3	Ig M	B cells
—	MB2	P	4	IG G1	B cells
CD3	UCHT1	F	5	Ig G1	Peripheral T cells
CD3	CD3 (poly)	P	1	—	Peripheral T cells
—	βF1	P/F	6	Ig G1	β chain of T cell receptor
—	bcl-2 protein	F	2	Ig G	B cells, T cells
CD15	Dako M1	P	1	Ig Mκ	HRS cells
CD30	BerH2	P	1	Ig G1	Ki-1 activation antigen
CD45RB	PD7/26—2B11	P	1	Ig G1	Leukocyte common antigen

P = paraffin sections; F = frozen sections; 1 = DAKO; 2 = Dr D. Y. Mason, Oxford, UK; 3 = ICN, Biomedicals; 4 = Eurodiagnostics; 5 = Prof. P. Beverley, ICRF, London, UK; 6 = Laboratory Impex Limited, London.

Table 2. *Immunophenotype of HRS Cells: Frozen Sections**

ST	n	%	CD19	CD20	CD22	CD3	βF1
NS	14	>50	0	1	2	0	0
		10–50	6	5	3	2	1
		<10	4	6	3	2	2
		(0)	(4)	(2)	(6)	(10)	(11)
MC	6	>50	2	1	0	0	0
		10–50	3	3	3	0	0
		<10	0	2	1	1	0
		(0)	(1)	(0)	(2)	(5)	(6)
TO	20		15 (75%)	18 (80%)	12 (60%)	5 (25%)	3 (15%)

* Staining patterns: CD19, CD20: surface; CD22: cytoplasmic; CD3: cytoplasmic (1 case surface); βF1: cytoplasmic (1 case membrane).
ST = subtype; n = number of cases; % = percentage of immunoreactive HRS cells; NS = NSHD; MC = MCHD; TO = Total.

cases HRS cells expressed at least one B-cell antigen (Figure 1a–c) with all three B-cell antigens being expressed in 50% of cases and two in a further 25%. CD19 and CD20 expression was seen on the surface of various numbers of HRS cells, whereas CD22 expression was confined to the cytoplasm as is appropriate for B cells in other than mature stages of differentiation.[38] As can be seen from Table 2, in most cases less than 50% of HRS cells were reactive with the B-cell markers. T-cell antigens were expressed by a minority of HRS cells in only 5 (25%) cases (Figure 1d) and in only one of these were the antigens expressed appropriately (i.e., on the cell surface/membrane). In two of these five cases, the HRS cells also expressed all three B-cell antigens, with expression of two in a further two cases and a single B-cell antigen in one case. In each frozen section, a minority of HRS cells showed moderate-to-strong cytoplasmic reactivity for bcl-2 protein.

In paraffin sections a variable number of HRS cells in each case were reactive with CD30. In 43/55 (78%) of cases CD15 positive HRS cells were identified. Using CD30 and CD15 HRS cells displayed membrane staining and/or a cytoplasmic paranuclear dotlike reactivity. No reactivity of HRS cells was observed with CD45

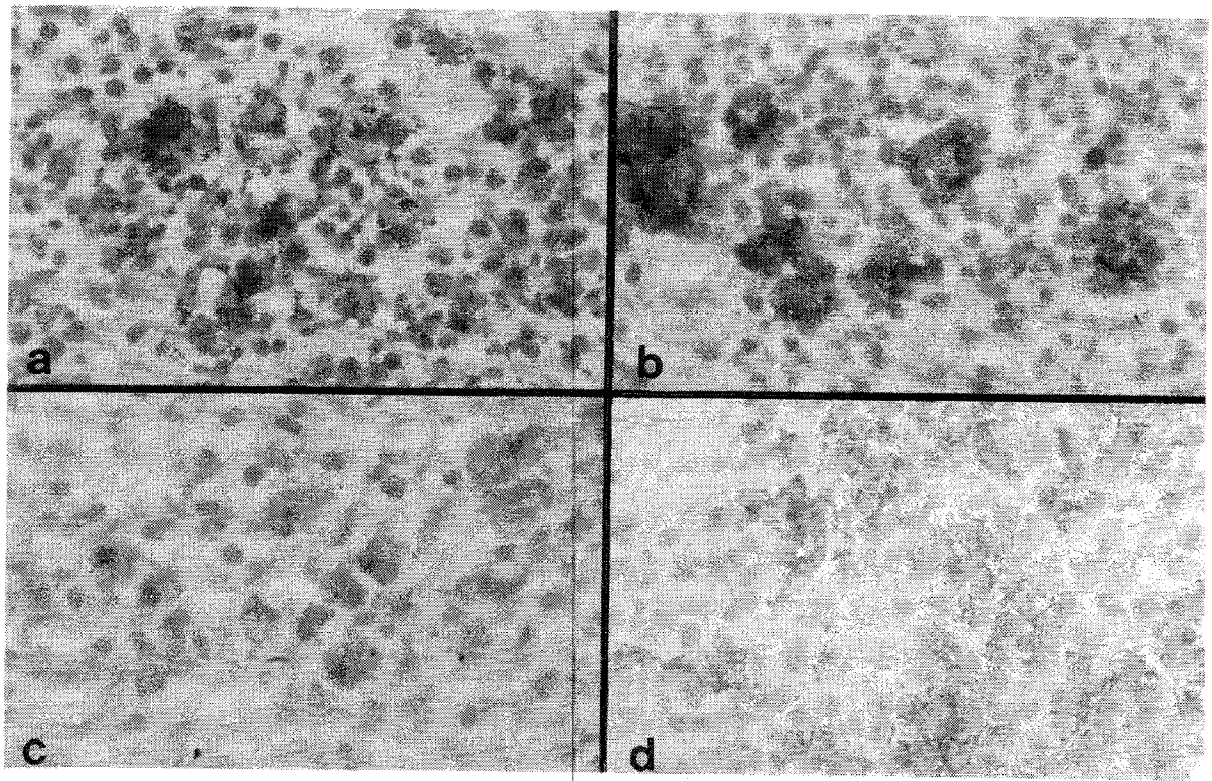

Figure 1. *Frozen sections from cases of Hodgkin's disease stained* (a) *with CD19,* (b) *with CD20,* (c) *with CD22 and* (d) *with CD3. There is strong surface membrane staining of HRS cells with the B cell markers CD19 and CD20 while CD22 stains the cytoplasm. CD3 staining is confined to the cytoplasm (immunoalkaline phosphatase).*

Table 3. *Immunophenotype of HRS Cells: Paraffin Sections**

ST	n	%	CD20	CDw75	MB2	CD3	βF1
NS	45	>50	11	5	1	0	0
		10–50	4	8	4	1	0
		<10	10	9	7	4	0
		(0)	(20)	(23)	(33)	(40)	(45)
MC	10	>50	0	1	0	0	0
		10–50	5	3	5	0	0
		<10	2	4	1	0	0
		(0)	(3)	(2)	(4)	(10)	(10)
TO	55		32 (58%)	30 (55%)	18 (33%)	5 (9%)	0

* Staining patterns: CD20: surface; CDw75: surface and/or cytoplasmic; MB2, CD3: cytoplasmic.
ST = subtype; n = number of cases; % = percentage of immunoreactive HRS cells; NS = NSHD; MC = MCHD; TO = Total.

(CD30, CD15 and CD45 were not applied on frozen sections). Using antibodies to B and T cells in paraffin sections (Table 3) HRS cells expressed at least one B-cell antigen in 42/55 (76.4%) cases (Figure 2a, b, c). In 32 (58%) of these, HRS cells strongly expressed the B-cell specific antigen CD20 whereas in the remaining 10 cases they expressed B–cell-associated antigens recognized by the antibodies CDw75 or MB2. Again, as in frozen sections less than 50% of HRS cells were reactive with B-cell markers in most of the cases. Weak-to-moderate expression of CD3 antigen (Figure 2d) was detected in the cytoplasm of occasional (a single cell in two cases) HRS cells in five cases in all of which the cells expressed at least one B-cell antigen. None of the HRS cells in paraffin sections were reactive with βF1.

Molecular Genetics

Of 18 cases of HD analyzed by Southern blotting only one (NSHD) showed rearrangement of the Ig heavy chain gene which was present in DNA digested with each of the three restriction enzymes. Using the polymerase chain reaction (PCR), no rearrangement of the bcl-2 gene (major breakpoint) was detected in any of the 19 frozen and 32 paraffin cases.

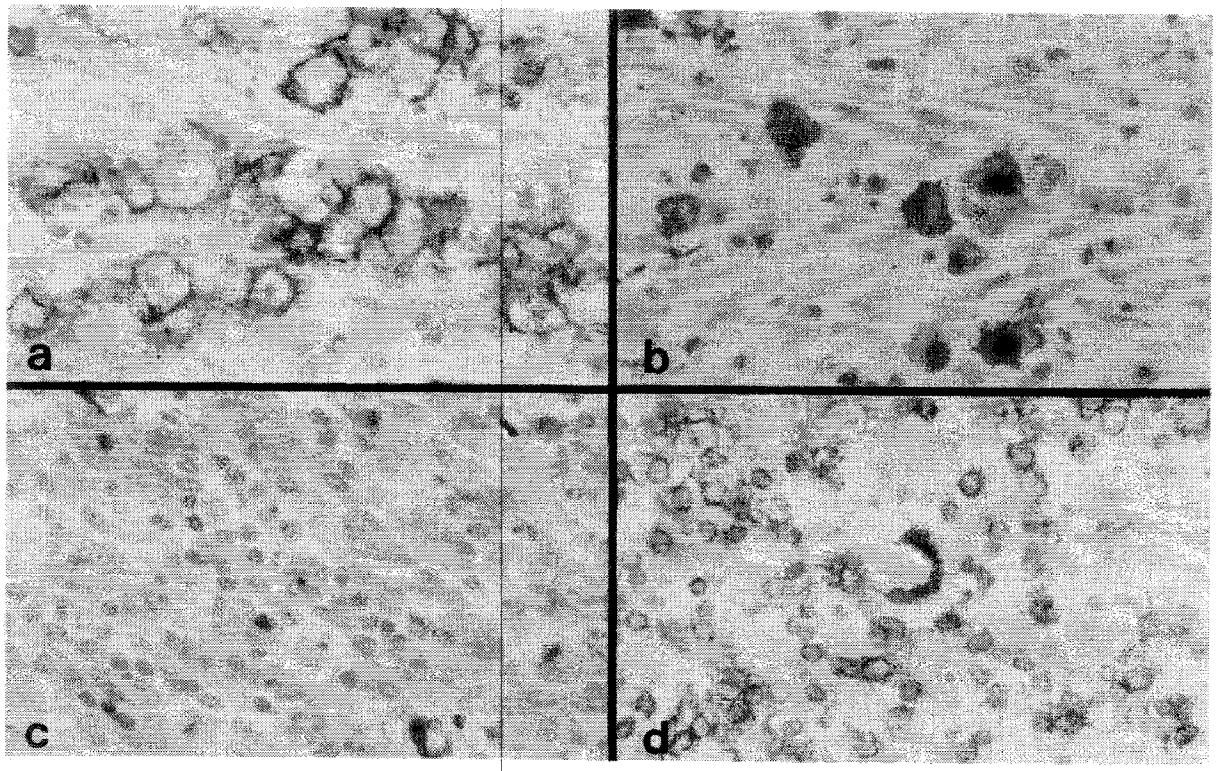

Figure 2. *Paraffin sections from cases of Hodgkin's disease stained* (a) *with CD20,* (b) *with CDw75,* (c) *with MB2 and* (d) *with CD3. The B-cell markers CD20, CDw75, and MB2 clearly label HRS cells. CD3 staining is confined to the cytoplasm of HRS cells in contrast to surface membrane labelling of surrounding T lymphocytes (immunoperoxidase).*

Discussion

Following the work of Stein et al.,[10] who described the constant expression by HRS cells of the lymphoid activation antigen CD30, there has been a broad consensus that these cells are derived from lymphocytes. Opinions are, however, divided as to whether HRS cells are derived from B cells, T cells, or both. Most immunohistochemical studies have favored a T-cell origin.[20,22–25] Many of these immunohistochemical studies are flawed in respect of the small number of cases studied,[20,23] a failure to include B-cell markers,[22,24] or to report their results obtained with B-cell markers.[25] Furthermore, the suggested T-cell origin of HRS cells in immunohistochemical investigations is mainly based on descriptions of intracytoplasmic, rather than appropriate membrane expression, of T-cell markers such as CD3.[11,20,24] Although cytoplasmic CD3 expression does not completely exclude a T-phenotype, cytoplasmic CD3 expression, as far as we are aware, has not been described in T-cell lymphomas. Strong evidence that aberrant cytoplasmic CD3 expression can occur in B cells is provided by Cibull et al.[24] who reported CD3 expression in RS (presumably the L & H variant) cells in three cases of nodular lymphocytic predominant HD. A B-cell phenotype of the L & H variant RS cells in this disease is well recognized[39] and Cibull et al.[24] argue for the aberrant nature of CD3 staining in their cases. In this respect, we have observed positive cytoplasmic staining with CD3 in at least 30% of the cells in three B-cell lymphoblastic cell lines, whereas the B-cell markers used in our study did not stain any cells in five T-lymphoblastic cell lines (unpublished observations). Molecular genetic investigations of Hodgkin's disease, although compromised by the small numbers of tumor cells present in the tissue, have tended to favor a B-cell derivation of HRS cells.[13,14,17,26–28] When selected cases of HD were used (either with high content of RS cells, or following HRS cell enrichment by cell separation techniques, Ig gene rearrangement could be detected in a higher percentage of cases.[13,14,40] Recent descriptions of the integration of Epstein–Barr virus (EBV) in HRS cells[41–46] provide further evidence in favor of a B-cell origin although in rare instances EBV has been reported to occur in T-cell lymphomas.[47,48] In addition, the frequent association of Hodgkin's disease with B-cell non-Hodgkin's lymphoma[49–51] in contrast to only occasional reports of HD with associated T-cell lymphomas[51,52] implies a relation between Hodgkin's disease and B cells.

Our immunohistochemical findings provide evidence in support of a B-cell origin of HRS cells. However, in most cases, a minority of HRS cells expressed B-cell antigens, which is different from non-Hodgkin's B-cell lymphomas in which almost all the tumor cells express B-cell markers. This finding was also observed in the study of Pinkus et al.,[53] who found a variable number of L26 (CD20) positive HRS cells in 34/63 (54%) of their investigated cases. Using the antibody L26, which recognizes the CD20 antigen in frozen and paraffin-embedded tissue,[54] membrane staining of HRS cells was detected in 76.4% of our cases overall and in 90% of cases in which frozen sections could be studied. Not surprizingly, this indicates a degree of antigen loss resulting from tissue fixation and processing. Antibody L26, which was not used in most of the previous immunohistochemical studies of Hodgkin's disease, is a particularly avid B-cell specific reagent in both frozen and fixed tissue.[16] If the other B-cell restricted markers, CD19 and CD22, are included, in 80% of our cases HRS cells show an unequivocal B-cell phenotype. If the results with the B-cell associated markers MB2 and CDw75 are included, in 87% of our cases of Hodgkin's disease the HRS cells showed a B-cell phenotype. Our results using T-cell markers are comparable to those of other workers showing cytoplasmic reactivity with T-cell markers in a minority of HRS cells.[11,20,24] Significantly in each case in which T-cell markers were positive B-cell antigens were also expressed.

With the Southern-blotting technique, we were able to determine a B-cell genotype in only one case of Hodgkin's disease (NSHD). This may reflect insufficient sensitivity of Southern blotting when the neoplastic population accounts for only a minority of cells in the tissue and is common to all previous studies of Hodgkin's disease using this technique.[11,17,26–28] Alternatively, the paucity of evidence of Ig gene rearrangement in Hodgkin's disease may reflect the role of EBV in its pathogenesis, for which there is increasing evidence.[42,44–46] With rare exceptions,[47,48] EBV selectively infects B-lymphocytes and in EBV-induced B-lymphoblastic cell lines, the cells, while continuing to express B-cell surface antigens, often do not show evidence of rearranged Ig genes.[55] Lack of sensitivity cannot explain our inability to confirm the findings of Stetler–Stevenson et al.[19] who demonstrated bcl-2 gene rearrangement in Hodgkin's disease with PCR. Said et al. were likewise unable to confirm these findings but these authors used a slightly less sensitive method.[28] Our PCR method is of similar sensitivity to that used by Stetler–Stevenson et al., and we have no explanation for this discrepancy. Bcl-2 protein expression in malignant lymphomas, which was present in only a minority of HRS cells in each of our cases, has been shown not to be dependent on the presence of a t(14;18) translocation.[56,57] Although we did not find differences in levels of bcl-2 expression in HRS cells compared with surrounding small lymphocytes, such differences might be seen in paraffin material or cytocentrifuged cell preparations, since differences in staining

intensity have been observed in tumor cells of follicular non-Hodgkin's lymphomas.[56] There is a high Ki-67 score in HRS cells in Hodgkin's disease,[20,58] and bcl-2 expression in only occasional HRS cells may be explained by the failure of those cells in cycle to express the protein.[57]

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Monoclonal Antibody Studies in B(Non-T)-Cell Malignancies

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Tumor cells suspensions prepared from 129 B- or non-T cell malignancies were investigated with a panel of 10 monoclonal antibodies and conventional surface marker techniques. Surface immunoglobulin (slg) and Bl antigen proved to be the most useful markers for B-cell lineage. Six major subtypes of acute lymphoblastic leukemia (ALL) of non-T cell nature are now recognized by these immunological techniques, including null-ALL, Ia-ALL, lymphoid stem cell ALL, pre-pre-B ALL, pre-B ALL and B-ALL.

In cases of chronic leukemias and lymphomas of non-T cell nature, 80% of the tumor was defined by slg and 88% by Bl antigen as definitely of B-cell lineage. The clonal character was also defined in 68% of the tumor on the basis of the detection of predominant single light chain in slg. Ia-like antigen was detected in almost all cases (96%).

Leukemic cells from all cases of chronic lymphocytic leukemia (CLL), chronic lymphosarcoma cell leukemia (CLsCL) and hairy cell leukemia (HCL) reacted with OKIal and anti-Bl, and leukemic cells from most of them with anti-pan T monoclonal antibody (10.2). In more than half of CLL and CLsCL, leukemic cells were reactive with J5, OKM1, 9.6 and OKT8, but not with OKT3, OKT4 and OKT6. HCL cells had almost the same reactivity with these monoclonal antibodies as CLL and CLsCL cells except that J5 remained unreactive. These results indicated that Japanese CLL, CLsCL and HCL were different from Western ones at least with respect to surface marker characteristics.

In cases of lymphomas, heavy chains of sIg were expressed in polyclonal fashion, especially in follicular lymphoma and diffuse lymphomas of medium sized cell type and large cell type, indicating that lymphomas of these types may originate from follicular center cells of the heavy chain switching stage. Anti-T monoclonals were also reactive with lymphoma cells. In about half of follicular lymphomas and diffuse lymphomas of the medium sized cell type, lymphoma cells reacted with 10.2, and less frequently with 9.6, OKT4 and OKT3. On the other hand, only in one or two cases of diffuse lymphoma of the large cell type and of immunoblastic sarcoma (IBS), did tumor cells react with 10.2 and 9.6, but this was exceptional. In more than 25% of IBS, tumor cells also reacted with OKT8, but not with 0KT4 and OKT3. These results indicated that anti-T monoclonals are no longer specific for T-cell lineage. It must be recognized that, in B- or non-T cell lymphoma as well as chronic leukemia, tumor cells are sometimes reactive with several anti-T monoclonals. These results can cause confusion. Therefore, it is still necessary to perform conventional marker studies in addition to monoclonal antibody studies in the case of B- or non-T cell malignancies. Further development of useful anti-B monoclonals is strongly desired.

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Monoclonal Antibody 1F5 (Anti-CD20) Serotherapy of Human B Cell Lymphomas

By Oliver W. Press, Frederick Appelbaum, Jeffrey A. Ledbetter, Paul J. Martin, Joyce Zarling, Pamela Kidd, and E. Donnall Thomas

Four patients with refractory malignant B cell lymphomas were treated with continuous intravenous (IV) infusions of murine monoclonal antibody (MoAb) 1F5 (anti-CD20) over five to ten days. Dose-dependent levels of free serum 1F5 were detected in all patients. Two patients had circulating tumor cells and in both cases 90% of malignant cells were eliminated from the blood stream within four hours of initiation of serotherapy. Antigenic modulation did not occur, and sustained reduction of circulating tumor cells was observed throughout the duration of the infusions. Serial bone marrow aspirations and lymph node biopsies were examined by immunoperoxidase and immunofluorescence techniques to ascertain MoAb penetration into extravascular sites. High doses (100 to 800 mg/m^2/d and high serum 1F5 levels (13 to 190 μg/mL) were required to coat tumor cells in these compartments in contrast to the low doses that were adequate for depletion of circulating cells. Clinical response appeared to correlate with dose of MoAb administered with progressive disease (52 mg), stable disease (104 mg), minor response (1,032 mg), and partial response (2,380 mg) observed in consecutive patients. The patient treated with the highest 1F5 dose achieved a 90% reduction in evaluable lymph node disease, but the duration of this remission was brief (six weeks). This study demonstrates that high doses of 1F5 can be administered to patients with negligible toxicity by continuous infusion and that clinical responses can be obtained in patients given >1 g of unmodified antibody over a ten-day period.

MONOCLONAL ANTIBODY (MoAb) serotherapy of malignancy represents a theoretically attractive, potentially nontoxic approach for the treatment of neoplastic disease.[1-3] Preliminary animal experimentation has demonstrated both the effectiveness and limitations of MoAbs that recognize tumor-associated antigens in preventing growth of murine hematologic malignancies.[4,5] Early human trials have shown that infusion of antibodies recognizing lymphoid cell differentiation antigens is a well-tolerated therapy capable of coating tumor cells and causing tumor regression in some patients.[6-8] However, the antitumor effectiveness of MoAbs has been limited by the presence of circulating free antigen, antigenic modulation, development of human antimouse antibodies (HAMA), emergence of antigen-negative tumor cell variants, and the inadequacy of host effector cell mechanisms.[5,8-11]

Here we present findings in four patients with B cell lymphomas treated with a murine IgG2a MoAb (1F5) chosen to avoid many of the previously encountered obstacles. MoAb 1F5 recognizes a 35,000 dalton antigen (Bp35, CD20) present on the surface of normal and malignant B cells[12] that is not shed from the cell surface (unpublished observations), does not modulate in response to MoAb binding, and does not bind to any other normal tissues. Consequently, prolonged continuous MoAb 1F5 therapy can be administered without inducing the unresponsiveness to therapy that has necessitated intermittent bolus therapy in previous trials.[8-11] We have administered 1F5 by continuous intravenous (IV) infusion (52 to 2,380 mg over five to ten days) to determine toxicity, kinetics, penetration to extravascular tissues, and efficacy. Our studies have shown 1F5 to be a minimally toxic therapy capable of depleting circulating tumor cells at low doses and lymph node tumor cells at high doses. However, responses were transient, suggesting that antibodies conjugated to toxins or radioisotopes might afford more lasting clinical benefit than unmodified antibody.

MATERIALS AND METHODS

Antibody preparation. Murine MoAb 1F5 (IgG2a) was produced in BALB/c mice and purified as previously described.[13] Antibody 1F5, along with the B1 antibody,[15] has been assigned to the CD20 (anti-Bp35) cluster group by the Second International Workshop on Human Leukocyte Differentiation Antigens.[14] The reactivity of antibody 1F5 with normal and malignant B cells has previously been reported.[13,16,17] MoAb 1F5 was purified from ascites by saturated ammonium sulfate precipitation followed by diethyl aminoethyl (DEAE)-Sephacyl (Pharmacia, Piscataway, NJ) column chromatography.[16] Testing of the purified antibody by Microbiological Associates (Bethesda, MD) has shown it to be free of bacterial, viral, or endotoxin contamination. Preclinical testing in two macaques (*M. fascicularis*) injected with 1F5 IV showed that this antibody was capable of eliminating circulating B cells and penetrating lymph nodes without causing any acute toxicity (J. Ledbetter, unpublished observations, 1983). A battery of normal human autopsy tissues was screened for reactivity with antibody 1F5 by an indirect immunoperoxidase method. No reactivity was seen with any tissue except those known to be rich in B lymphocytes (tonsils, lymph nodes, spleen). Tissues failing to bind 1F5 included heart, thyroid, adrenal, lung, muscle, kidney, testis, skin, colon, breast, and brain.

Patient selection. Adult patients with histologically confirmed B cell lymphomas shown by immunoperoxidase or immunofluorescence techniques to be reactive with the 1F5 antibody were eligible

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for this study if they had failed previous conventional therapy (chemotherapy and/or radiotherapy), if they had normal renal and hepatic function (creatinine <2.0 mg/dL, bilirubin <1.5 mg/dL), had evaluable disease, had not received any other treatment for ≥four weeks, had no other active medical problems, and signed an informed consent approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center.

Study design. Prestudy blood, marrow, and lymph node specimens were obtained and analyzed by conventional histopathology and by an indirect immunoperoxidase method (Vectastain, Vector Laboratories, Raritan, NJ) for evidence of tissue involvement with tumor cells capable of binding 1F5. Cell suspensions of these tissues were analyzed by two-color flow cytometry using a panel of fluorescein and phycoerythrin antibody conjugates[13] to determine the baseline immunologic phenotypes of the resident cell populations (see below). Intradermal skin testing with 10 μg of antibody 1F5 in 0.1 mL of normal saline was performed, and no hypersensitivity responses were observed. Allopurinol (300 mg/d) was given throughout antibody administration. A bolus loading dose was given over one to two hours IV to rapidly achieve steady state serum antibody levels. The loading dose was calculated from the following equation: Loading dose = 1.4 × elimination half-time (in days) × daily maintenance dose.[18] Preclinical studies of murine anti-CD20 antibody infusions in nonhuman primates (Ledbetter, unpublished data) and clinical trials of murine MoAbs administered to patients with graft-ν-host disease (GVHD)[19] suggested an elimination half-time of 1.2 days, and this figure was used in calculating the loading doses. Patients were assigned a predetermined maintenance antibody dose that was diluted in 500 mL normal saline and administered by continuous IV infusion for five to ten days. (Patient 1 had premature discontinuation of his infusion after five days due to rapidly progressive lymphoma.) The maintenance antibody doses administered to the patients are summarized in Table 1. The dose escalation range was chosen to progress from safe low doses (5 mg/m²/d) known to be well tolerated for other MoAbs[6,7,9,10,19] to high doses (400 to 800 mg/m²/d), which we felt were more likely to result in good tissue penetration. We initially planned to escalate doses between patients. However, because of the absence of toxicity, poor penetration of low doses of antibody into patients 1 and 2, and slow patient accrual, doses were escalated progressively in patients 3 and 4 (from 10 mg/m²/d to 800 mg/m²/d) to achieve high circulating antibody levels that we felt would be more likely to achieve extravascular tissue penetration.

Table 1. Summary of Patient Characteristics

Patient	1	2	3	4
Age/Sex	42/M	64/M	63/M	45/M
Type of Lymphoma	DML	WDLL	DPDL	DHL
Stage	IVB	IVA	IVA	IVB
Total Dose of IF5	52.4 mg	104.8 mg	1,032 mg	2,380 mg
Duration of Therapy	4.5 days	10 days	10 days	7 days
Response	Progression	Stable Disease	Minor Response	Partial Response

Abbreviations: DML, diffuse mixed small and large cell lymphoma; WDLL, diffuse well-differentiated lymphocytic lymphoma (small lymphocytic, working formulation); DPDL, diffuse, poorly differentiated lymphocytic lymphoma (diffuse small cleaved cell, working formulation); DHL, diffuse "histiocytic" lymphoma (diffuse large cell lymphoma, working formulation).

Patient monitoring. Pretreatment tests included a history and physical examination, relevant radiographic studies and computed tomographic scans, chemistry batteries, uric acid levels, complete blood cell counts (CBCs) and differentials, prothrombin time, partial thromboplastin time, serum complement levels (CH50, C4, C3), immune complex levels (C1Q binding assay), urinalysis, ECG, and cell surface marker analysis. Patients were examined twice daily during antibody infusion. Serial serum specimens for 1F5 levels and antimouse antibody levels, blood counts, chemistries, and blood specimens for surface marker studies were obtained four hours after initiation of 1F5 therapy and daily thereafter. Patients were discharged at the termination of antibody infusions. Blood samples were obtained on an outpatient basis for the above tests on days 1, 2, 7, and 21 after cessation of therapy and monthly thereafter. Serum complement and immunoglobulin levels were tested pretreatment and on days 1, 5, and 10 and then at roughly monthly intervals for six months.

Response criteria. Standard response criteria were employed as follows: Complete response-disappearance of all measurable and evaluable disease; Partial response; reduction by ≥50% of leukemic cell counts and ≥50% reduction in the size of a measurable lesion, and no increase in the size of any measurable or evaluable lesions or appearance of new lesions; Stable disease: Less than a partial response without an increase of >25% in leukemic cell count and <25% increase in any measurable lesion. Progression: Increase in leukemic cell count (>25%), appearance of new lesions, or an increase of 25% or greater in any measurable lesion.

Measurement of free 1F5 and human antimouse antibody. Serum 1F5 levels and human antimouse antibody levels (HAMA) were measured by solid phase competitive inhibition radioimmunoassay (RIA) as previously described.[19]

Detection of cell-bound 1F5. Assessment of tumor cell coating by infused antibody 1F5 was accomplished on serial specimens of peripheral blood, bone marrow, and lymph nodes by indirect immunoperoxidase and immunofluorescence techniques. Peripheral blood and bone marrow mononuclear cells were obtained by Ficoll-Hypaque (LSM, Litton Bionetics, Kensington, MD) density gradient centrifugation. Lymph node biopsies were divided in thirds: one portion was minced into a single cell suspension, another part was fixed in formalin for routine histologic staining, and another portion was frozen in liquid nitrogen for immunohistologic staining. Lymph node frozen sections were fixed to gelatin-coated glass slides and stained with rabbit antimouse immunoglobulin (Vectastain, Vector Laboratories, Raritan, NJ) using an indirect avidin-biotin technique. Pretreatment biopsies served as controls.

Cell suspensions of blood, bone marrow, and lymph nodes from antibody-treated patients were examined by flow cytometry (FACS IV, Becton Dickinson, Sunnyvale, CA) for the presence of surface 1F5 by using fluorescein-conjugated goat antimouse immunoglobulin (FITC-GAMIg; TAGO, Burlingame, CA). The mean fluorescence intensity of cells stained with FITC-GAMIg was compared to the intensity of cells incubated with excess 1F5 in vitro before staining to assess the saturation of binding sites in vivo. Relative CD20 surface antigen density was estimated for normal and malignant B cells by measuring the mean fluorescence intensity of cells stained in vitro with saturating quantities of fluorescein-conjugated anti-CD20 antibody after correcting for nonspecific fluorescence with a control reagent.[13,17]

Tumor cell surface antigen phenotypes were determined by both immunofluorescence and immunoperoxidase methods using peroxidase, fluorescein, or phycoerythrin conjugates of MoAbs 10.2 (anti-CD5), HB10a (anti-DR), G1–4 or 3E10 (antikappa), and 2C3 (anti-μ) as previously described.[13,17] Serial monitoring of these tumor cell markers demonstrated that CD20-negative tumor cells were not

generated during 1F5 therapy by antigenic modulation (data not shown).

CASE HISTORIES

Patient 1 was a 42-year-old man with stage IVB diffuse, mixed, small and large cell lymphoma who presented in 1982 with fever, diffuse lymphadenopathy, and hepatosplenomegaly. Previous therapy included CHOP (cyclophosphamide, adriamycin, vincristine, and prednisone) chemotherapy, intrathecal methotrexate, whole brain irradiation, splenectomy, sequential upper and lower hemibody irradiation, and four cycles of bleomycin, cytosine arabinoside, vincristine, procarbazine, and prednisone. He was referred for 1F5 therapy in July, 1984 because of refractory disease. He did not respond to low-dose 1F5 and was taken off study after five days of infusion because of progressive bone marrow (BM) and liver replacement with tumor. Salvage CHOP chemotherapy was given, but the patient died of progressive lymphoma on March 16, 1985.

Patient 2 is a 64-year-old man with stage IVA diffuse, small, lymphocytic lymphoma diagnosed by lymph node (LN) and BM biopsy in 1976. He received multiple chemotherapeutic regimens (CVP, CHOP, chlorambucil, and CCNU, etoposide, and methotrexate) with partial responses. He was referred for 1F5 serotherapy in December 1984, 11 months after his last course of chemotherapy. He did not respond to low dose 1F5 but had stable disease that did not require therapy until the summer of 1985 when he was begun on bleomycin, etoposide, BCNU, and Decadron, to which he remains partially responsive.

Patient 3 was a 63-year-old man with stage IVA diffuse, small, cleaved-cell lymphoma involving lymph nodes, marrow, and spleen. Previous therapy included splenectomy, chlorambucil, and CVP. He was referred for 1F5 therapy in December 1984, one month after his last cycle of CVP because of the development of refractory disease with rapidly progressive adenopathy and lymphocytosis (>30,000 cells/μL). He showed a minor response to intermediate dose 1F5 therapy. ProMACE/MOPP chemotherapy was given in January and February 1985 without response. A partial response occurred after therapy with high-dose cytosine arabinoside, but the patient died with marrow aplasia in July 1985.

Patient 4 was a 45-year-old man with sclerosing, diffuse large-cell lymphoma presenting in January 1983 with bowel and lymph node involvement. Therapy included eight cycles of CHOP, intrathecal methotrexate, involved field abdominal radiation, prophylactic cranial irradiation, and allogeneic marrow transplantation (in March 1984). He was referred for 1F5 therapy because of refractory lymphoma in late October 1985. He had been on dexamethasone (4 mg/d) for many months as symptomatic therapy for myalgias, and this was continued during serotherapy. After treatment with 1F5 there was a partial response that lasted six weeks. He then redeveloped progressive lymphoma and refused further treatment. He expired on December 21, 1985.

RESULTS

Serum 1F5 levels. Circulating free antibody levels were detectable by RIA in all patients throughout the period of infusion (Fig 1). Patients 1 and 2 received MoAb doses of 5 mg/m^2/d and consistently had 1F5 serum concentrations of 0.3 to 1.0 μg/mL. Patients 3 and 4 received escalating antibody doses and had corresponding increases in 1F5 levels. For comparable antibody doses the patients with circulating antigen-positive tumor cells (1 and 3) had lower serum levels of 1F5 than the patients who did not have significant numbers of circulating malignant lymphocytes (2 and 4, see Fig 1), probably reflecting the effect of antibody

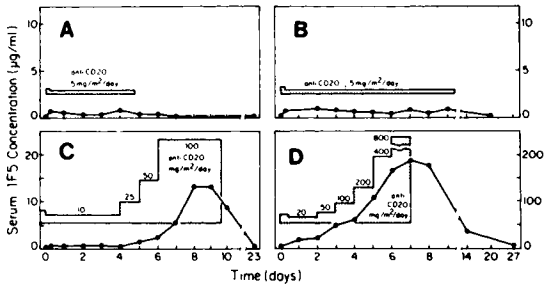

Fig 1. Serum 1F5 concentrations in patients receiving continuous infusions of antibody. MoAb 1F5 concentrations were determined by solid phase radioimmunoassay. The doses of 1F5 infused in each patient are indicated by the superimposed stippled bars. (A), patient 1; (B), patient 2; (C), patient 3; (D), patient 4.

binding to tumor cells. Antibody remained detectable in the serum for as long as three weeks after termination of infusion in patient 4 (peak concentration 190 μg/mL). However, 1F5 levels fell into the undetectable range within three days of termination of infusion in patients 1 and 2 (peak levels 1 μg/mL) and within two weeks in patient 3 (peak level 13.4 μg/mL). Rough estimates of the serum elimination half-times were calculated to be 24 hours for patient 1 (from 0.93 μg/mL to .22 μg/mL in the 48 hours after termination of infusion), 42 hours for patient 3 (from 13.4 μg/mL to 9.0 μg/mL in 24 hours), and 52 hours in patient 4 (from 179 to 35.6 μg/mL over five days). Data for patient 2 were insufficient for estimation of a serum half-life. These elimination half-times are in good agreement with previous studies of murine anti-CD20 antibodies in nonhuman primates (J. Ledbetter, unpublished results) and studies of murine anti-T cell antibodies in patients with GVHD.[19]

CD20 antigen density on tumor cells. Table 2 summarizes the relative Bp35 surface antigen densities on patient lymphoid cells from blood, bone marrow, and lymph nodes as determined by direct immunofluorescent analysis.[13,17] The density of this antigen on normal B lymphocyte populations is also listed for comparison. Patients 1 and 4 had CD20 densities on their malignant cells comparable to those seen on normal, resting B lymphocytes (eg, peripheral blood B cells and tonsil mantle zone B cells[13]). Patient 3 had a much higher surface antigen density on his lymphoma cells, comparable to that observed on normal, activated B cells (tonsil germinal center cells[13]). Patient 2 had a very low Bp35 antigen density on his lymph node and bone marrow tumor cells with a mean fluorescence intensity only 3.5 times higher than control cell populations lacking the antigen. (This degree of staining was unequivocally greater than control, however.) Patients 2 and 4 had negligible numbers of circulating tumor cells morphologically, confirming the negligible staining with FITC-1F5 seen by immunofluorescence (nearly all circulating lymphocytes were T cells in these patients). Of interest, the bone marrow of patient 4 was grossly involved with tumor but failed to bind FITC-1F5, suggesting that an antigen-negative tumor cell variant was responsible for infiltration of this tissue. With this single exception, the different sites of lymphomatous involvement within a given patient showed similar CD20 antigen densi-

Table 2. Relative CD20 Antigen Densities on Normal and Malignant Lymphoid Cells

Cell Type	Relative Antigen Density*
Normal Tissues	
1. Peripheral Blood T cells	2†
2. Peripheral Blood B cells	82
3. Tonsil Mantle Zone B cells	70
4. Tonsil Germinal Center B cells	256
Lymphoma Patients	
1. Patient 1‡	
a. Blood lymphocytes	96
2. Patient 2	
a. Bone marrow	7
b. Lymph node cells	7
3. Patient 3	
a. Blood lymphocytes	301
b. Bone marrow cells	301
c. Lymph node cells	235
4. Patient 4	
a. Blood lymphocytes (uninvolved)	2
b. Bone marrow cells	2
c. Lymph node cells	84

*Expressed as the linear channel number of the mean fluorescence intensity measured on a FACS IV cell sorter for cells stained with saturating concentrations of fluorescein-conjugated anti-CD20 antibody by the method of Ledbetter and Clark.[13]

†Negative control (unstained) cells also showed a mean fluorescence intensity of 2.

‡Patient 1 had an unaspirable marrow and no accessible adenopathy, so immunofluorescent studies were done solely on circulating malignant cells.

ties. No definitive conclusion regarding the clinical responsiveness of tumors bearing different surface CD20 densities is possible because of the small number of patients treated and variable antibody doses administered.

Effects of 1F5 on peripheral blood lymphocytes. Two patients (1 and 3) had appreciable numbers of circulating malignant cells. In both patients antibody administration resulted in an immediate decrease in the number of circulating tumor cells (assessed by morphological criteria and by surface immunologic phenotypes). Patient 1 had an 86% decline in the number of blood lymphoma cells (from 1.27 × $10^3/\mu L$ to 0.18 × $10^3/\mu L$) within four hours of institution of 1F5 therapy. Patient 3 had a 91% decrement in circulating tumor cells (from 18.21 × $10^3/\mu L$ to 1.61 × $10^3/\mu L$) in the same brief time interval (Fig 2). These effects were obtained with low doses of antibody in both patients (5 mg/m^2 and 10 mg/m^2, respectively) and were sustained throughout the entire period of infusion (five and ten days). Flow cytometry of circulating PBL stained with FITC-GAMIg demonstrated saturation of 1F5 antibody binding sites on tumor cells in both patients (although complete saturation in patient 3 was only achieved at the higher dose of 100 mg/m^2/d, Fig 3). Serial tumor cell surface-antigen phenotyping (using two-color immunofluorescence with reagents recognizing other tumor-associated antigens [see Materials and Methods]) demonstrated that antigenic modulation did not occur (data not shown). In both patients termination of 1F5 therapy was accompanied by a rapid reappearance of

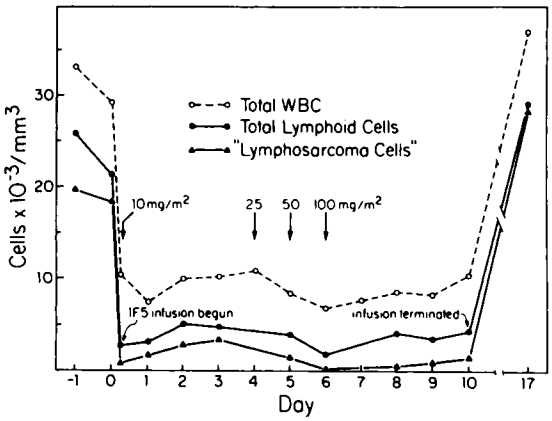

Fig 2. Depletion of circulating lymphoma cells in patient 3 during infusion of MoAb 1F5.

circulating tumor cells that reached pretreatment levels within two to three days (Fig 2).

Effects of 1F5 on bone marrow tumor cells. Patients 1, 2, and 3 had evaluable marrow involvement with lymphoma. In patients 1 and 2, antibody doses of 5 mg/m^2/d were not sufficient for saturation of 1F5 antibody binding sites on tumor cells in the marrow. Patient 3 received escalating doses of 1F5 in conjunction with serial marrow aspirations to estimate the amount of antibody required for coating of tumor cells in the marrow. Serial fluorescence histograms (Fig 4) clearly showed that an antibody dose of 10 mg/m^2/d was insufficient (4% saturation of Bp35 binding sites) but that 100 mg/m^2/d could produce significant coating (61% saturation of Bp35 binding sites) of marrow tumor cells.

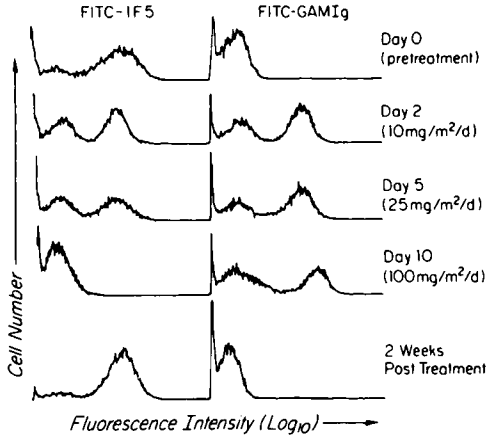

Fig 3. In vivo labeling of circulating tumor cells with 1F5 antibody in patient 3 as assessed by serial flow cytometry of peripheral blood lymphocytes with FITC-GAMIg (to detect mouse antibody 1F5 bound to tumor cells in vivo) and FITC-1F5 (to detect unoccupied Bp35 [CD20] binding sites). Pretreatment PBL stained brightly with FITC-1F5 because of abundant free CD20 sites on circulating lymphoma cells. Serial histograms on days 2, 5, and 10 revealed coating of PBL with 1F5 (detected with FITC-GAMIg). Saturation of binding sites is shown on day 10 by absence of unoccupied Bp35 receptors capable of binding FITC-1F5. By two weeks posttreatment, bound murine MoAb was no longer detectable on PBL.

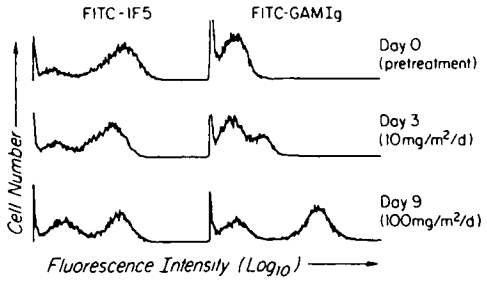

Fig 4. Penetration of MoAb 1F5 into bone marrow of patient 3 during serotherapy as assessed by flow cytometry of mononuclear cells from BM aspirates using FITC-GAMIg (to detect cell-bound 1F5) and FITC-1F5 (to detect unbound Bp35 sites).

Regression of marrow lymphoma was not seen in any of these three patients.

The marrow of patient 4 was unusual in that it appeared to contain tumor cells that did not express the Bp 35 antigen. Although marrow aspirates and biopsies contained unequivocal large cell lymphoma, no tumor cells reactive with FITC-1F5 were detected by flow cytometry. In contrast, tumor cells in cervical and inguinal lymph nodes had the same morphology as the cells in the marrow but reacted strongly with antibody 1F5 as assessed by both immunoperoxidase and immunofluorescence techniques (see Table 2). As would be anticipated, infusion of antibody 1F5 had no effect on the antigen-negative tumor cells in the marrow of this patient.

Effects of 1F5 on lymph nodes. Patients 2, 3, and 4 had evaluable adenopathy that was biopsied before treatment and on the last day of antibody infusion. Immunoperoxidase and immunofluorescent analyses showed no penetration of antibody 1F5 into the nodes of patient 2 (who received 105 mg over ten days). There was minor perivascular penetration detectable only by immunoperoxidase methods in patient 3 (1,032 mg over ten days). In contrast, significant coating of tumor cells detectable by both immunoperoxidase and immunofluorescence (Figs 5 and 6) was present in patient 4 (2,380 mg over seven days) with 69% saturation of available binding sites. In vitro studies showed that an ambient 1F5 antibody concentration of 24 μg/mL was necessary to achieve 69% saturation of cell-surface binding sites. Since the serum 1F5 concentration in patient 4 at the time of his lymph node biopsy was approximately 190 μg/mL, we estimate that a 1F5 antibody gradient of 8:1 existed between serum and lymph node interstitial fluid.

No clinical response was observed in the nodes of patient 2. Some inguinal nodes regressed by 25% in patient 3, but most lymph nodes were unaffected. There was marked regression of all nodes in patient 4 with a calculated >90% reduction in tumor burden (Fig 7). Of note, the diminution of LN size did not begin until day 5 of antibody infusion, and progressive node shrinkage continued for three weeks after cessation of 1F5 infusion. The response duration was brief, however, with regrowth of LN occurring six weeks after therapy.

Overall clinical response. Patient 1 had diminution of

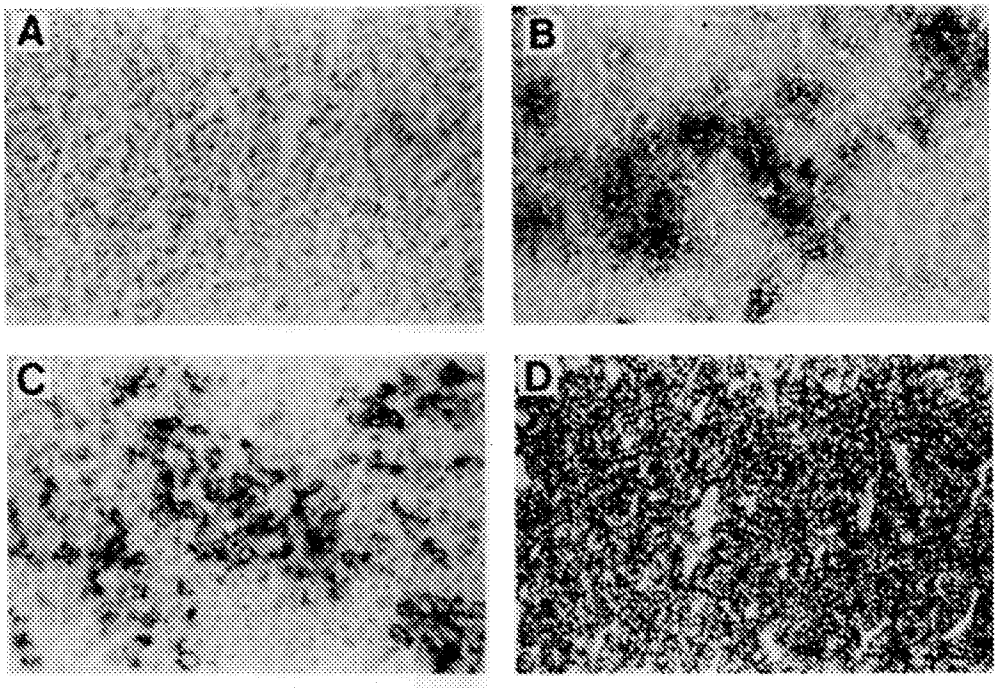

Fig 5. Indirect immunoperoxidase staining of lymph node frozen sections with GAMIg to detect in vivo labeling of tumor cells during murine MoAb 1F5 serotherapy (original magnification × 250). (A) Patient 2: LN biopsy performed on day 10 of therapy while receiving 5 mg/m²/d of antibody 1F5. Negligible staining indicates absence of penetration of LN by antibody at this dose. (B) Patient 3: LN biopsy performed on day 9 of therapy while receiving 100 mg/m²/d of 1F5. Staining of tumor cells in perivascular locations is present. (C) Patient 4: LN biopsy performed on day 10 of therapy while receiving 800 mg/m²/d of 1F5. Peroxidase staining is appreciable at this dose, although the distribution remains heterogeneous. (D) Saturation of 1F5 binding sites in patient 3 by in vitro incubation of an LN section with excess 1F5 antibody. The section shown in this figure was from the same LN biopsy depicted in Fig 4B.

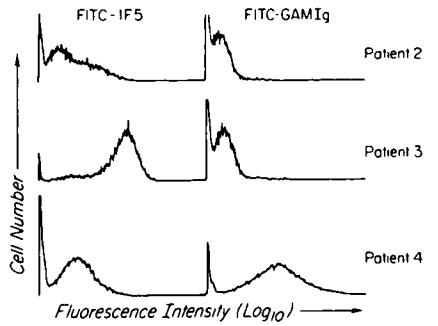

Fig 6. Analysis of 1F5 penetration into LN during serotherapy assessed by flow cytometry of LN suspensions. FITC-GAMIg was used to detect in vivo coating of tumor cells with antibody 1F5. Patient 2 had only a small subpopulation of LN cells possessing the Bp35 antigen (shown with FITC-1F5). No penetration of 1F5 into LN could be shown with FITC-GAMIg. Virtually all LN cells in patient 3 possessed the Bp35 antigen and stained intensely with FITC-1F5, but poor in vivo penetration of 1F5 had occurred as shown by absence of FITC-GAMIg staining. (Immunoperoxidase staining of LN sections was capable of demonstrating some penetration of 1F5 into this LN, however [see Fig 5B]). Patient 4 had significant penetration of 1F5 into LN as shown by staining with FITC-GAMIg.

circulating tumor cells, but progressive liver and marrow lymphoma required premature termination of 1F5 therapy and institution of salvage chemotherapy. Patient 2 had no response of his evaluable marrow or LN disease. Patient 3 had a minor response consisting of transient 90% reduction of circulating tumor cells and 25% shrinkage of some but not all LN but no response in the marrow. Patient 4 had a partial response consisting of 90% reduction of all evaluable LN.

Toxicity. No clinically significant toxicity was observed in any of our patients. Patients 1 and 4 had asymptomatic, intermittent, low-grade fever (38 to 39 °C) lasting six days and two hours, respectively. Transient decrements of platelet and neutrophil counts to 50% to 75% of baseline levels were observed in all four patients. These changes were rapidly reversible in all cases except patient 1, where progressive cytopenias were clearly due to marrow replacement with tumor. In the other three instances, the blood counts stabilized after one to two days and often demonstrated some recovery, even before discontinuation of antibody infusion. No bleeding or infectious episodes occurred during antibody treatment. No allergic, pulmonary, renal, hepatic, or cutaneous sequelae occurred. Renal function as measured by 24-hour creatinine clearance was unchanged after completion of antibody therapy. Complement consumption was observed in patients 1 (40% reduction of CH_{50} and C4 levels) and 3 (97% reduction of CH_{50}, 94% reduction of C4 levels), but circulating immune complexes could not be detected at any time in any patient.

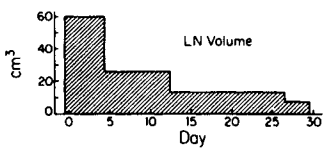

Fig 7. Reduction in tumor burden in patient 4. A 90% reduction in LN volume was observed over a four-week period.

Effects of 1F5 on normal B cells. Attempts to monitor numerical changes and functional alterations of normal B cells in patients receiving 1F5 serotherapy (by assessing proliferative responses to polyclonal B cell mitogens and in vitro Epstein-Barr virus (EBV)-induced immunoglobulin synthesis) were precluded by the extremely low numbers of normal B cells that could be harvested from these patients following antibody infusion. The paucity of B cells was due to two factors: baseline deficiency of normal B lymphocytes (a common feature of advanced, refractory B cell malignancies) and depletion of normal as well as malignant B cells by 1F5 therapy (which has also been documented in normal nonhuman primates infused with anti-CD20 antibodies, [Jeffrey Ledbetter, unpublished results]). Quantitative immunoglobulin levels were carefully monitored in all patients before, during, and for up to five months after 1F5 therapy. Baseline hypogammaglobulinemia was present in patients 1 and 3, but no patient demonstrated a decline in any subclass of immunoglobulin consequent to 1F5 therapy.

Human antimouse antibody levels. IgM HAMA were undetectable by RIA in all patients. Low levels of IgG HAMA (2 × pretreatment control levels) became detectable five months after serotherapy in patient 1 only.

DISCUSSION

This report summarizes our findings in four patients with refractory malignant B cell lymphomas treated with MoAb 1F5 (anti-CD20) by continuous IV infusion for five to ten days. Our study differs from previous serotherapy trials of hematologic malignancies by employing an antibody directed against a nonmodulating antigen. This feature allowed us to maintain continuous high serum antibody levels without inducing the tumor refractoriness generally encountered with modulating antigens.[8-11,20] The continuous infusion mode of administration allowed delivery of very high doses of antibody (up to 800 mg/m^2/d in patient 4) without the significant pulmonary toxicity that is often observed following bolus injection of high MoAb doses.[8,10,21] Serial kinetic measurements revealed a dose-dependent relationship between the amount of antibody infused and the concentration of free MoAb in the blood stream. We found that even low doses of 1F5 (5 to 10 mg/m^2/d) were capable of depleting circulating tumor cells from the blood stream analogous to observations made using murine MoAbs T101 (for chronic lymphocytic leukemia) and J5 (for acute lymphocytic leukemia).[9-11,20] However, in contrast to the studies with modulating antibodies T101 and J5 given by prolonged or repeated administration, the responses induced by the nonmodulating 1F5 were sustained throughout the duration of the infusion (five to ten days).

Although small doses of 1F5 sufficed to deplete circulating tumor cells, penetration of antibody into extravascular sites such as bone marrow and lymph nodes proved to be much more problematic. Intravenous administration of 400 to 800 mg/m^2/d was required to achieve 69% saturation of binding sites on lymph node tumor cells. Even at these doses the intranodal distribution of antibody was heterogeneous. The immunoperoxidase staining patterns observed in LNs

suggested passive diffusion of antibody down a concentration gradient from small blood vessels into the LN parenchyma. The clinical responsiveness observed appeared to correlate with 1F5 dose administered, peak-serum MoAb concentration achieved, and degree of extravascular tissue penetration obtained. A total dose of 52.4 mg was associated with progressive disease (patient 1), 104.8 mg resulted in stable disease in patient 2, 1,032 mg caused a minor response in patient 3, and 2,380 mg produced a partial response in patient 4.

These observations have been made on a very small group of patients, each of whom had a different type of B cell lymphoma. The degree to which our findings might be applicable to other lymphoma patients is unclear, since each patient has a different tumor burden and distribution, different numbers of circulating malignant cells acting to absorb infused MoAb, and different surface densities of the Bp35 antigen. Consequently, the dose levels and kinetic data found in our patients can only serve as a rough guideline for the management of other patients with this antibody. Generalizations from our findings with 1F5 to other MoAbs should be made with caution in light of recent studies demonstrating dramatic, unpredictable kinetic and functional differences among different antibodies recognizing the same antigen.[22]

The toxicity seen in these four patients was insignificant. Minor fever and moderate cytopenias were the only adverse effects observed despite administration of massive doses of antibody 1F5 to patients 3 and 4. Since neither platelets nor neutrophils express the Bp35 antigen or label with FITC-1F5, the exact mechanism for the decrement in neutrophil and platelet counts is uncertain. It is of interest that similar decrements in blood counts have also been observed in patients treated with anti-idiotypic antibodies.[8] Small quantities of antibody might be absorbed via Fc receptors to these cells, which may then be removed from the circulation by the reticuloendothelial system. Although the development of HAMA has been a major problem in some reported series,[8,23] antimouse antibodies were detected in only one of our four patients, and in this patient they did not appear until five months after 1F5 therapy was completed. These findings are in accord with other studies demonstrating that patients with B cell malignancies undergoing monoclonal serotherapy seldom develop HAMA, whereas patients with T cell malignancies or solid tumors receiving similar treatment often develop antimouse antibodies.[24,25]

Previous trials of MoAb serotherapy have also generally encountered minimal toxicity.[3] The major adverse events described to date involved anaphylactoid reactions in patients with large circulating tumor cell burdens and/or high circulating antigen levels given high doses of antibody by rapid bolus injection.[8,10,21] These episodes have been ascribed to pulmonary leukostasis resulting from sequestration of antibody-coated tumor cells in the pulmonary vasculature leading to wheezing, dyspnea, and hypotension. The absence of circulating antigen and the prolonged duration of antibody administration in our trial were mitigating factors that probably helped avoid these untoward sequelae in our patients.

The relative merits of continuous infusion of MoAbs compared with intermittent bolus therapy remain debatable. For many antibodies continuous infusion is not feasible because of antigenic modulation. In such circumstances intermittent therapy is necessary to allow regeneration of cell surface antigen. The bolus method is less cumbersome than continuous infusion and achieves higher peak MoAb concentrations for equivalent doses. Whether the maintenance of uniform high-circulating antibody levels and reduction in toxicity achievable with continuous infusion are sufficiently advantageous to offset the inconveniences remains unanswered. Nevertheless, maintenance of steady state antibody levels in this trial has afforded an advantageous setting for kinetic measurements and for the assessment of the serum concentrations required for MoAb penetration into the extravascular space.

The mechanisms by which unmodified MoAbs might cause elimination of tumor cells in vivo remain controversial. Most workers currently view antibody-dependent cellular cytotoxicity and reticuloendothelial system phagocytosis of MoAb-coated cells as the most likely processes involved.[3,26] Murine MoAbs (including 1F5) fix human complement poorly in vitro, and consequently complement-mediated tumor cell lysis is not thought to be of major significance in vivo. The significant consumption of complement in two of our four patients was unexpected and suggests a possible role for complement in eliminating tumor cells.

The short, incomplete responses obtained with serotherapy using unmodified MoAbs have been of minimal clinical benefit (with the notable exception of the patient described by Miller et al).[7] Consequently, innovative MoAb administration schedules, testing of new antibodies, and administration of antibody conjugates will be necessary if the promise of monoclonal serotherapy is to be realized. Badger et al have already convincingly demonstrated cures of lymphomas in mice treated with radioiodinated MoAbs in a setting in which unmodified MoAbs were ineffective.[27] Recent clinical reports of responses in patients with Hodgkin's disease or hepatoma treated with radioiodinated antiferritin antibodies suggest that this approach will also be useful in man.[28,29] Radiolabeled MoAbs can potentially kill not only the tumor cells to which they bind but could also kill neighboring cells that do not bind antibody by virtue of poor tissue penetration, antigenic modulation, or somatic mutation ("antigen-negative variants"). Toxin-antibody conjugates are similarly promising,[30] although antigens such as CD20, which are not endocytosed after ligand binding,[31] might not be good targets for this approach, since immunotoxin internalization is generally required for cell killing.[32] The findings of our current pilot study should assist in the rational design of subsequent trials employing such radiolabeled or toxin-conjugated immunotoxins.

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Treatment of refractory non-Hodgkin's lymphoma with radiolabeled MB-1 (anti-CD37) antibody.

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The biodistribution, toxicity, and therapeutic potential of anti-CD37 monoclonal antibody (MoAb) MB-1 labeled with iodine 131 (131I) was evaluated in ten patients with advanced-, low- or intermediate-grade non-Hodgkin's lymphomas who failed conventional treatment. Sequential dosimetric studies were performed with escalating amounts of antibody MB-1 (0.5, 2.5, 10 mg/kg) trace-labeled with 5 to 10 mCi 131I. Serial tumor biopsies and gamma camera imaging showed that the 10 mg/kg MoAb dose yielded the best MoAb biodistribution in the ten patients studied. Biodistribution studies in the five patients with splenomegaly and tumor burdens greater than 1 kg indicated that not all tumor sites would receive more radiation than normal organs, and these patients were therefore not treated with high-dose radioimmunotherapy. The other five patients did not have splenomegaly and had tumor burdens less than 0.5 kg; all five patients in this group showed preferential localization and retention of MoAb at tumor sites. Four of these patients have been treated with 131I (232 to 608 mCi) conjugated to anti-CD37 MoAb MB-1, delivering 850 to 4,260 Gy to tumor sites. Each of these four patients attained a complete tumor remission (lasting 4, 6, 11+, and 8+ months). A fifth patient, whose tumor did not express the CD37 antigen, was treated with 131I-labeled anti-CD20 MoAb 1F5 and achieved a partial response. Myelosuppression occurred 3 to 5 weeks after treatment in all cases, but there were no other significant acute toxicities. Normal B cells were transiently depleted from the bloodstream, but immunoglobulin (Ig) levels were not affected, and no serious infections occurred. Two patients required reinfusion of previously stored autologous, purged bone marrow. Two patients developed asymptomatic hypothyroidism 1 year after therapy. The tolerable toxicity and encouraging efficacy warrant further dose escalation in this phase I trial.

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REVIEW

Immunologic Classification of Leukemia and Lymphoma

By Kenneth A. Foon and Robert F. Todd, III

Important insights into leukocyte differentiation and the cellular origins of leukemia and lymphoma have been gained through the use of monoclonal antibodies that define cell surface antigens and molecular probes that identify immunoglobulin and T cell receptor genes. Results of these studies have been combined with markers such as surface membrane and cytoplasmic immunoglobulin on B lymphocytes, sheep erythrocyte receptors on T lymphocytes, and cytochemical stains. Using all of the above markers, it is now clear that acute lymphoblastic leukemia (ALL) is heterogeneous. Furthermore, monoclonal antibodies that identify B cells, such as the anti-B1 and anti-B4 antibodies in combination with studies of immunoglobulin gene rearrangement, have demonstrated that virtually all cases of non-T-ALL are malignancies of B cell origin. At least six distinct subgroups of non-T-ALL can now be identified. T-ALL is subdivided by the anti-Leu-9, anti-Leu-1, and antibodies that separate T lymphocyte subsets into three primary subgroups. Monoclonal antibodies are also useful in the subclassification of non-Hodgkin's lymphoma, and certain distinct markers can be correlated with morphologic classification. The cellular origin of the malignant Reed-Sternberg cell in Hodgkin's disease remains uncertain. A substantial number of investigators favor a myelocyte/macrophage origin based on cytochemical staining; however, consistent reactivity with antimonocyte reagents has not been demonstrated. Although monoclonal antibodies are useful in distinguishing acute myeloid from acute lymphoid leukemias, they have less certain utility in the subclassification of acute myelogenous leukemia (AML). Attempts to subclassify AML by differentiation-associated antigens rather than by the French-American-British (FAB) classification are underway in order to document the potential prognostic utility of surface markers. Therapeutic trials using monoclonal antibodies in leukemia and lymphoma have been reported. Intravenous (IV) infusion of unlabeled antibodies is the most widely used method; transient responses have been demonstrated. Antibodies conjugated to radionuclides have been quite successful in localizing tumors of <1 cm in some studies. Therapy trials with antibodies conjugated to isotopes, toxins, and drugs are currently planned. Purging of autologous bone marrow with monoclonal antibodies and complement in vitro has been used in ALL and non-Hodgkin's lymphoma; preliminary data suggest that this approach may be an effective therapy and may circumvent many of the obstacles and toxicities associated with in vivo monoclonal antibody infusion.

RECENT ADVANCES in immunology have led to important insights into leukocyte differentiation and the cellular origin of leukemia. It is now possible to define stages of human lymphocyte and granulocyte differentiation precisely using highly specific monoclonal antibodies that define cell surface antigens and molecular probes that identify rearrangement of immunoglobulin and T cell receptor genes. These can be combined with more traditional cell markers such as surface membrane (SmIg) and cytoplasmic immunoglobulin (CIg) on B lymphocytes, sheep erythrocyte receptors on T lymphocytes, and cytochemical stains. In this review, we summarize advances in the classification of leukemia and lymphoma and their importance in our understanding of normal leukocyte differentiation and therapeutic implications.

CELL MARKERS

B lymphocytes. B lymphocytes are usually identified by the presence of SmIg. Progenitors of B lymphocytes, commonly referred to as "pre-B cells," are present in fetal liver and normal bone marrow; the cells display cytoplasmic μ-heavy chain (Cμ) but lack intracytoplasmic light chain and SmIg. B and pre-B lymphocytes may also have receptors for the third component of complement (C'3) and for the Fc portion of IgG. Fc and C'3 receptors are not specific for the B cell lineage and are found in other cells such as monocytes and some nonhematopoietic cells. Similarly, histocompatibility-related antigens (Ia or HLA-DR) are also found on the surface of B cells, but are not unique to them.[1-4] Plasma cells are the most mature B lymphocytes; they lack detectable

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Table 1. Monoclonal Antibodies Reactive with Human B Lymphocytes

Antibody (Subclass)	Pattern of Reactivity	Mol wt of Antigen (kd)	Cluster Designation	Reference
BA-1 (IgM)	B lymphocytes, granulocytes malignant B cells	45,55,65	CD24	4,5
FMC1 (IgM)	B lymphocytes, malignant B cells	NR	NA	6
FMC7	< 50% B lymphocytes, some malignant B cells	NR	NA	7
Anti-B1 (IgG_2)	B lymphocytes, malignant B cells	35	CD20	8
Anti-B2 (IgM)	B lymphocytes, malignant B cells (receptor for Epstein-Barr virus and C3d)	140	CD21	9,10
Anti-B4 (IgG_1)	B lymphocytes, malignant B cells	40	CD19	11
Anti-B5 (IgM)	Activated B lymphocytes, malignant B cells	75	NA	12
P1153/3	B lymphocytes, malignant B cells	NR	NA	13
OKB1 (IgG_1)	B lymphocytes, malignant B cells	168	NA	14,15
OKB2 (IgG_1)	B lymphocytes, granulocytes	NR	NA	14,15
OKB4 (IgM)	B lymphocytes, malignant B cells	87	NA	14,15
OKB7 (IgG_{2a})	B lymphocytes, malignant B cells	175	NA	14,15
Anti-HLB-1 (IgG_{2a})	B lymphocytes, malignant B cells	NR	NA	16
41H.16 (IgG_{2a})	B lymphocytes, malignant B cells	39	NA	17
Anti-BL1 (IgG_1)	Subpopulation of B lymphocytes, malignant B cells, granulocytes	Glycolipid	NA	15
Anti-BL2 (IgG_{2a})	B lymphocytes, malignant B cells, activated T cells	68	NA	15,19
Anti-BL3 (IgG_1)	Subpopulations of B lymphocytes, some malignant B cells, plasma cells, activated T cells	105	NA	15
Anti-PCA-1 (IgG_{2a}) and anti-PCA-2 (IgG_1)	Plasma cells, malignant plasma cells, weakly on monocytes and granulocytes	NR	NA	20
Anti-PC-1 (IgM)	Plasma cells, malignant plasma cells	28	NA	21
LN-1 (IgM)	B lymphocytes, malignant B cells, epithelial tumors	Sialoantigen	NA	22
LN-2 (IgG_1)	B lymphocytes, malignant B cells (nuclear membrane and cytoplasm)	35	NA	22
HD6, HD39, 29-110 SJ10-1H11, SHCL-1	75% of B lymphocytes, most malignant B cells	135	CD22	23
MNM6, PL-13, Blast-2	Germinal center B cells, not on resting B cells, some malignant B cells	45	CD23	23

NR, not reported, NA, not applicable.

The anti-B series, anti PC-1, and anti PCA-1 are available through Coulter Immunology, Hialeah, Fla; BA-1 through Hybritech Inc, San Diego; and the OKB series through Ortho System, Inc, Raritan NJ.

Table 2. Monoclonal Antibodies Reactive with Human T Lymphocytes

Antibody	Pattern of Reactivity	Mol wt of Antigen (kd)	Cluster Designation	Reference
OKT1, anti-T1, anti-Leu-1, 10.2 (Lyt-2), SC-1, A50, T101	Pan-T lymphocyte, pan-thymocyte	65	CD5	26-34
OKT3, anti-T3, anti-Leu-4, UCHT1	Pan-T lymphocyte (mitogenic)	20,20,25	CD3	27,28,35,36
Anti-Ti	Anti-clonotypic (T cell antigen receptor)	49-51 (α) 43 (β)	NA	37-39
12.1, T411	Pan-T, subpopulation of B	120	CD6	40,41
OKT11, anti-T11, anti-Leu-5, 9.6 (Lyt-3)	Pan-T lymphocyte (sheep erythrocyte receptor)	40-50	CD2	42-44
3A1, anti-Leu-9 (4H9), WT1, 4A	Pan-T lymphocyte	40	CD7	45-48
OKT4, anti-T4, anti-Leu-3	T helper/inducer	55	CD4	26-28,49-52
Anti-TQ1	Subset of T inducer cells	NR	NA	53
OKT5, OKT8, anti-T8, anti-Leu-2	T cytotoxic/suppressor	32-43	CD8	26,27,51,52,54,55
OKT6, NA1/34, anti-Leu-6	Thymocytes	45	CD1	27,28,56
OKT9, 5E9	Thymocytes, lymphoblasts, monocytes (anti-transferrin)	90	NA	27,28,57
OKT10	Thymocytes	45	NA	27
Anti-Ta_1	Activated T lymphocytes	105	NA	58
Anti-Tac	Interleukin-2 receptor	55	CD25	59-61

NR, not reported, NA, not applicable.

The OKT series of antibodies is available through Ortho Systems, Inc, Raritan, NJ; Leu series through Becton Dickinson Co, Mountainview, Calif; anti-T through Coulter Immunology, Hialeah, Fla; Lyt through New England Nuclear, Boston; and T101 through Hybritech Inc, San Diego.

SmIg but have CIg. Unlike the CIg found in pre-B lymphocytes, CIg in plasma cells includes both heavy and light chains.

A number of heteroantisera and, more recently, monoclonal anitbodies that identify B cell-associated antigens have been described (Table 1).[4-22] Where applicable, the nomenclature and clusters of differentiation (CD) defined by the Second International Workshop on Human Leukocyte Differentiation Antigens are shown.[23-25]

T lymphocytes. T lymphocytes were initially identified by their ability to bind sheep erythrocytes spontaneously. T lymphocytes also react with T cell-specific antisera and anti-T cell monoclonal antibodies, which may also be used to identify T lymphocytes, and have proven to be more sensitive and discriminatory (Table 2).[26-61] Many of these antibodies react with immature T cells; others react with more mature T cells. Some of these antibodies identify antigens found on all T cells, whereas others occur only on T cell subsets.

Myeloid cells. Monoclonal antibodies to cell suface markers on peripheral blood myeloid cells and their bone marrow progenitors have been extensively investigated.[62-125] Some of these monoclonal antibodies detect antigens expressed by either peripheral blood monocytes or neutrophils. Other reagents identify surface markers common to monocytes and neutrophils; monocytes, neutrophils, and large granular lymphoid cells (LGL); monocytes and platelets; or neutrophils and LGLs (Table 3).

The expression of several monoclonal antibody-defined myeloid antigens corresponds to pathways of normal differentiation within the myeloid lineage. These antibodies are

Table 3. Representative Murine Monoclonal Antibodies That Identify Human Myeloid Cell Surface Antigens: Distribution of Antigen Expression Among Peripheral Blood Cells

Monocytes	Neutrophils	Neutrophils and Monocytes	Neutrophils, Monocytes, and Large Granular Lymphocytes	Monocytes and Platelets	Neutrophils and Large Granular Lymphocytes
Mo2 (CDw14,55)[62-64]*	B40.9[77]	MY7 (CDw13,160)[69]	Mo1 (CD11,155,94)†[62-64]	Mo4 (100)[63]	B73.1 (60-70)‡[112]
Mo3[93]	R1B19 (145,105)[77]	MY8[69]	OKM1 (CD11,155,94)†[106,107]	20.3 (CDw14)[104]	VEP13 (CD16)[113]
UC45 (45)[65]	82H5 (CD15)†[83]	Mo5 (CD11,94)[95]	OKM9 (CD11,155,94)†[108]	OKM5 (88)[108,110]	
UCHM1 (CDw14)[66]	80H.5[84]	B13.4[77,96]	OKM10 (CD11,155,94)†[108]	5F1 (CDw14,85)[98,103]	
UCHALF[66]	TG-1 (CDw15)[85]	B9.8[77,96]	B43.4[77,96]	MPA (135,93)[111]	
S16-144[67,68]	VIM-D5 (CD15,145,105)§[86]	B34.3[77]	αS-HCL 3 or anti-Leu M5(CDw14, 150, 95)[109]	SmO[88]	
MY3 (55)[59]	FMC 10 (CD15)§[87]	AML-2-23[91]			
MY4 (CDw14)[69]	FMC 12 (CD15)§[87]	PM-81[97]			
MY9[70]	FMC 13 (CDw15)[87]	1G10 (CD15)§[98]			
D5D6[71]	AHN-1 (145,105)§[88,89]	M206 (180)[99]			
C10H5[71]	MY-1§[90]	MMA or anti-Leu-M1[100]			
63D3 or antimonocyte .1 (200)[72,73]	PMN6[91]	S4-7 (150)[67,68]			
61D3 or antimonocyte .2 (75)[74]	PMN29[91]	AHN-7‖[101]			
	PMN 7C3¶[92]	80H.1[84]			
	3G8 (60-70)‡[93]	80H.3 (CDw15)[84]			
	1B5[94]				
	4D1 (59)[94]				
MOP-15 (CDw14)[75]		DUHL60.1 (CDw15)[102]			
MOP-9 or anti-Leu-M3 (CDw14)[75]		DUHL60.3 (CDw15)[102]			
Mac-120 or anti-Leu-M2(120)[76]		DUHL60.4 (CDw13)[102]			
		L4F3[103]			
B44.1 (55)[77]		T5A7 (CDw17)[103]			
1D5[78]		20.2 (CDw12)[104]			
PHM3 (50)[79]		VIM-2[105]			
4F2 (40,80)[80,81]					
FMC17 (CDw14)[82]					

*Antibody/antigen (cluster designation [CD], antigen mol wt, kd, reducing conditions), key references (superscript).

†Anti-C3bi receptor antibodies.

‡Anti-Fc receptor antibody.

§Antibodies bind to X-hapten, lacto-*N*-fucose-pentaosyl III.

‖Antibody immunoprecipitates broad band of 28 to 65 kd on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

¶Antibody immunoprecipitates two broad bands of 155 to 288 and 75 to 125 kd on SDS-PAGE.

Table 4. Representative Murine Monoclonal Antibodies That Identify Human Myeloid Differentiation Antigens

	S16-114[87,88]	MY9[70,114]	82H5[83]	MY10[115,116]	L1B2[103,117]	R1B19[87,88,77,89]	80H.5[84]	S4-7[87,88]	AHN-7[101]	L4F3[103,117]	T5A7[103,117]	Mo1[85,114,118]	Mo5[85]	MY7[114,118,120]	1G10[88,103,117]	PM81[87,114]	AML-2-23[81,114,121,122]	MY-1[80,118,123]	TG-1[68,85]	PMN 6[81,122]	MY8[85,118,119]	80H.3[84]	B13.4[77,85]	B34.4[77]	Mo2[83,114,124]	MY3/4[114,118,119]	20.3[104]	Mo4[83,124]	5F1[88,103]	SFL 23.6[125]
CFU-GEMM	+	+	+			−		−				−				−	−							−	−			−	−
CFU-GM (d 14)	+	+	+	+	+	−	+	+	+	+	−	−		+	+	+	−	−	−	−	−			−	−			−	−
CFU-GM (d 7)		+			+	+		+		+	+	−		+	+	+	+		−		−			−	−			−	−
Myeloblast	+	+		+	+	+	+	+	+	+	−	−	−	+	+	+	+	+		−	−	−	−	−	−	+	−	−	−
Promyelocyte	+	+	+	−	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	−	−	−	−	+	−	−	−
Myelocyte	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−
Metamyelocyte	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−
Neutrophil	−	−	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−
Monoblast/ promonocyte		+		+	+	−	−		−	+	+	+	+	+	−	+	+		−	−	+	+	+	+	+	+	+	+	+	−
Monocyte	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+	−
BFU-E	+	+	+	+	−	−	−	−		+	−	−		−	−	−	−		−	−	−					−			−	−
CFU-E	−	−			−	−	−	−		−	−	−		−	−				−		−					−			+	+
Erythroid precursor	−	−		−	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	−		−	−	−		+	−	+	+
Erythrocyte		−	−	−	−	−	−			−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
CFU-Mega			+							+																				
Megakaryocyte			−		−					−	−	−			−						−				−	−	+	+		
Platelet		−	−	−	−	−	−			−	−	−	−	−	−	−	−	−	−	−	−		−	−	−	−	+	+	+	−

therefore useful as tools for identifying hematopoietic cells at various stages of maturation and proliferative potential (Table 4 and Fig 1). Using either complement-dependent monoclonal antibody-mediated lysis (negative selection), or techniques such as fluorescence-activated cell sorting or immune rosetting (positive selection), it is possible to determine patterns of antigen expression by multipotent stem cells (CFU-GEMM) and by stem cells committed to the myeloid (CFU-GM), erythroid (BFU-E, CFU-E), or megakaryocyte/platelet (CFU-Mega) pathways of differentiation. In several reports, myeloid progenitor cells have been purified 50- to 100-fold from bone marrow mononuclear cells[85,126] enabling studies of morphologic and functional characteristics. Certain determinants are uniquely expressed by progenitor cells (eg, MY-10); other antigens are detectable on myeloid, erythroid, or platelet precursors corresponding to morphologically and histochemically distinct stages of maturation within the bone marrow. In the case of neutrophil differentiation, some antigens are either lost (Ia, MY-10) or acquired (Mo1, MY8, 80H.3, or B34.3) as cells progress from myeloblasts to mature neutrophils. Expression of other determinants (82H5, R1B19, S4–7) are maintained on all recognizable myeloid cells.

Although many of these antigenic determinants operationally serve as differentiation markers, some represent epitopes on functionally significant plasma membrane proteins, glycoproteins, and glycolipids. The glycoprotein heterodimer identified by the murine monoclonal antibodies Mo1 and OKM1 is a receptor for the binding of particles opsonized with C3 cleavage product C3bi (CR3 activity).[127,128] Antibodies B73.1 and 3G8 identify an Fc receptor for IgG that is expressed by LGL and/or neutrophils.[93,112] Data from immu-

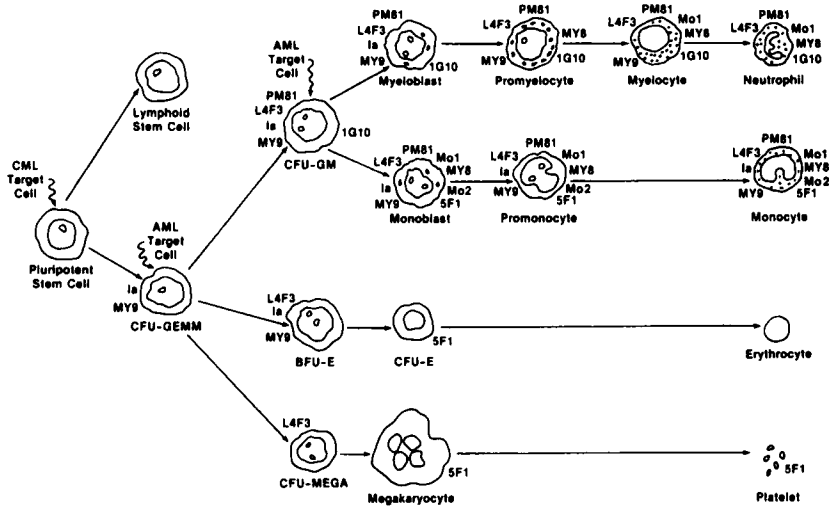

Fig 1. Schematic representation of human myeloid differentiation indicating the cell surface marker phenotype (as defined by selected well-characterized monoclonal reagents) of identifiable maturational steps. Phenotypic expression by monoblasts and promonocytes is tentative and based on positive expression by immature-appearing monocytoid cells in bone marrow. The phenotypes of CFU-GEMM, CFU-GM (early *or* late), BFU-E, CFU-E, and CFU-MEGA are based on the outcome of positive and/or negative selection (antibody-dependent, complement-mediated lysis, immunorosetting, or cell sorting) of human bone marrow cells on subsequent colony growth. Phenotypes of other cells are based on immunofluorescence analyses. Hypothesized sites of CML and AML leukemogenesis are indicated.

Table 5. Monoclonal Antibodies Leukemia-Associated Antigens

Antibody (Subclass)	Pattern of Reactivity	Molecular wt of Antigen (kd)	Cluster Designation	Reference
J5, BA-3, anti-CALLA	Most non-T-ALL, Burkitt's lymphoma, follicular lymphoma, some lymphoblastic lymphoma and T-ALL, rare normal cells	100	CD10	130-136
RFB-1	Myeloid progenitor cells, immature lymphoid bone marrow cells	NR	NA	141
BA-2, SJ-9A4 Du-ALL-1	Lymphohematopoietic bone marrow progenitor cells, most non-T-ALL, platelets	24	CD9	137-140,142
Anti-3-3	T-ALL	35-40	NA	143
Anti-3-40	T-ALL, some non-T-ALL, rare AML vimentin and keratin intermediate filaments in normal cells	35-40	NA	143,144
SN1	T-ALL	NR	NA	145
CALL2	T-ALL	NR	NA	146

NR, not reported; NA, not applicable.

J5 is available through Coulter Immunology, Hialeah, Fla; BA-2 and BA-3 through Hybritech Inc, San Diego.

noprecipitation or immunoblotting analyses indicate that several of these antibodies are specific for epitopes on the same parent structure or even the same epitope.

Leukemia-associated antigens. The common acute lymphoblastic leukemia-associated antigen (CALLA) was originally defined by antiserum produced in rabbits by immunization with SmIg-negative, sheep erythrocyte rosette–negative acute lymphoblastic leukemia (ALL) cells.[129] This antiserum reacted with a 100 kd glycoprotein antigen. Monoclonal antibodies that recognize CALLA (CD10) have recently been described (Table 5).[130,131] Although CALLA is absent on normal peripheral blood lymphocytes, it is not leukemia specific, and is present on normal terminal deoxynucleotidyl transferase (TdT) and Ia antigen–positive bone marrow cells that are thought to be lymphohematopoietic precursor cells.[132] CALLA has also been identified on renal tubular and glomerular cells, mammary epithelium, fetal small intestine epithelial cells,[133] granulocytes,[134] fibroblasts,[134] and melanoma cell lines.[135] CALLA is also present on Burkitt lymphoma cells, follicular lymphoma cells, and cells from 40% of patients with lymphoblastic lymphoma.[136]

P24/BA-2 is a second leukemia-associated antigen with a mol wt of 24 kd defined by the BA-2,[137] SJ-9A4,[138] and DU-ALL-1[139] monoclonal antibodies (CD9). These antibodies do not react with any normal circulating hematopoietic cells except for platelets. They react with most non-T ALL cells and, like CALLA, the P24/BA-2 antigen is present on ~3% to 9% of sheep erythrocyte receptor and SmIg negative TdT positive bone marrow mononuclear cells. This may also represent a bone marrow-derived lymphoid progenitor cell. The anti-J2 antibody[140] has a similar pattern of reactivity as do the BA-2, SJ-9A4, and DU-ALL-1 antibodies. The J2 antigen is also present on activated normal T lymphocytes and may react with the same surface molecule as BA-2. The RFB-1 antibody likewise reacts with immature lymphoid cells in the bone marrow,[141] but differs from BA-2 in its reactivity with myeloid progenitor cells.[141,142] The anti-3-3, anti-3-40,[143,144] SN1,[145] and CALL2[146] antibodies identify antigens found on T-ALL cells but not generally on non-T-ALL or other malignant or normal hematopoietic cells. A summary of the most frequently referenced antibodies and their cluster designations is presented in Table 6.

Immunoglobulin and T cell receptor genes. Recombinant DNA technology has provided important insights into antibody diversity and antigen-specific T cell receptors.[147,148] Immunoglobulins are composed of heavy and kappa and lambda light chains, encoded by genes on chromosomes 14, 2, or 22, respectively.[149-152] Immunoglobulin genes are encoded by discontinuous segments of DNA.[153-158] At one point in development, a potential antibody-producing cell must productively rearrange variable, diversity, and joining genes

Table 6. Frequently Referenced Antibodies with Cluster Designations

Cluster Designation	Antibody
CD1	OKT6, anti-Leu-6, NA1/34
CD2	OKT11, anti-T11, anti-Leu-5, 9.6
CD3	OKT3, anti-T3, anti-Leu-4, UCHT-1
CD4	OKT4, anti-T4, anti-Leu-3
CD5	OKT1, anti-T1, anti-Leu-1, 10.2, T101
CD6	12.1, T411
CD7	anti-Leu-9, 3A1, WT1, 4A
CD8	OKT5, OKT8, anti-T8, anti-Leu-2
CD9	BA-2, SJ-9A4, Du-ALL-1
CD10	J5, BA-3, anti-CALLA
CD11	Mo1/OKM1, Mo5
CDw12	20.2
CDw13	DUHL60.4, MY7
CDw14	Mo2, MY4, MOP-15, FMC 17
CD15	FMC10, VIM-D5, DUHL60.1
CD16	VEP13
CDw17	T5A7
CD19	anti-B4
CD20	anti-B1
CD21	anti-B2
CD22	SHCL-1, HD6, HD39, 29-110
CD23	PL13, MNM6, Blast-2
CD24	BA-1
CD25	anti-Tac

(VDJ), which are then linked to the constant region locus. Immunoglobulin gene rearrangements are hierarchical; μ heavy chain rearrangements precede light chain rearrangements, and κ light chain rearrangement precedes λ light chain rearrangements.[156-158] These rearrangements can be detected by Southern blot analyses of DNA from B cells using appropriately radiolabeled heavy or light chain probes. Heavy chain rearrangements have been identified in non-B cells, but light chain rearrangements appear to be restricted to B cells.[157,159,160] Clonal rearrangements of light chain genes are therefore an extremely sensitive tool to identify B cell malignancies.

The antigen-specific T cell receptor is a heterodimer formed by a 40- to 50-kd α subunit (Tα), and a 40- to 45-kd β subunit (Tβ).[161] It is associated with three 20- to 25-kd peptide chains identified by the T3 monoclonal antibody.[161] Recently, cDNA clones to the Tβ and Tα receptors have been isolated.[162-166] The human Tβ receptor gene has been localized to chromosome 7[167] and the human Tα receptor gene maps to chromosome 14.[168] The less well-defined Tγ receptor gene is currently under investigation. The T cell receptor genes undergo rearrangements in a fashion analogous to that of immunoglobulin genes. The Tγ receptor gene has been shown to be rearranged in murine cytotoxic T lymphocyte cell lines but not myeloma cell lines.[169] Similar to the Tβ chain gene, the Tγ gene rearrangement appears to occur early in T cell development, whereas Tα chain expression occurs later in thymic ontogeny.[170]

T_β gene rearrangements have been detected in malignant human T cells by Southern blotting.[171-173] This technique can detect as few as 1% tumor cells in a mixed cell population[171]; it is a sensitive diagnostic marker for T cell diseases. Rearrangements of the Tβ antigen receptor are reported in 25% of patients with non-T ALL,[174] and in a small proportion of patients with B cell leukemia.[175] This is similar to the rearrangement of immunoglobulin heavy chain genes in 10% of patients with T cell leukemia.[159,160]

Intracellular enzymes and biochemical markers. TdT is present in thymocytes and in a small percentage of bone marrow cells, but not in mature lymphocytes.[176,177] TdT is identified in all subtypes of ALL and is, therefore, not discriminative. TdT has also been demonstrated in a small proportion of acute myelogenous leukemia (AML) cells.[178] Other intracellular enzymes reported useful in identifying subsets of ALL include hexosaminidase, adenosine deaminase,[180] 5'-nucleotidase,[181] purine nucleoside phosphorylase,[182,183] and acid phosphatase.[184] Acid phosphatase is present in T-ALL cells but not in non-T-ALL cells. Cytochemical reactions are useful in the subclassification of AML.[185,186] The M1 through M3 myeloid subtypes contain myeloperoxidase and sometimes nonspecific esterase. Myelomonocytic leukemia cells (M4) also contain myeloperoxidase and nonspecific esterase; the latter is variably inhibited by sodium fluoride. Acute monocytic leukemia (M5) cells contain myeloperoxidase and nonspecific esterase which is completely inhibited by sodium fluoride.

Several surface membrane-associated biochemical markers of leukemia cells have also been described. The glycolipid asialo GM1 is found on cells from patients with ALL (non-T-ALL and T-ALL) but not on cells from patients with other forms of leukemia.[187] Alterations in membrane carbohydrates, such as decreased complex gangliosides,[188] carbohydrate-containing antigens,[189] and receptors for cholera toxin[190] have been reported on leukemia cells.

CLASSIFICATION OF THE LYMPHOID LEUKEMIAS AND LYMPHOMAS

Acute lymphoblastic leukemia. ALL is heterogeneous. The first surface markers used to differentiate subclasses of ALL were receptors for sheep erythrocytes,[191-193] which identify a T cell subset (15% to 20% of cases), and SmIg, which identifies a B cell subset (<5% of cases). Both T and B cell subgroups have an unfavorable prognosis.[194,195] The next important advance in identifying ALL was the development of an antiserum to CALLA.[129] CALLA reactivity identified a non-B, non-T subclass of ALL patients (~70% of cases) with a more favorable prognosis than T-ALL, B-ALL, or non-B, non-T-ALL without CALLA.[195] Other markers such as Ia antigen were commonly found on non-T-ALL and could help differentiate non-T-ALL from T-ALL.[196] By testing for Cμ heavy chain, a subset designated pre-B ALL has been identified.[197-199] Except for the presence of Cμ, this subset expresses the same surface markers as the CALLA form of non-T-ALL; it appears, however, to have a less favorable prognosis.[200]

With the development of monoclonal antibodies, it became evident that the T cell subset of ALL was heterogeneous.[201-205] More recently, studies employing immunoglobulin gene rearrangements and monoclonal antibodies that identify B cell-associated antigens have demostrated that most cases of non-T-ALL are derived from the B cell lineage.[156,206-208] We review these data and present a new classification for ALL based on these recent observations.

Non-T-ALL. Two important areas of research have prompted a reassessment of non-T-ALL. First, monoclonal antibodies that recognize B cell-associated antigens have been identified; many are present on non-T-ALL cells. The most specific of these antibodies is probably anti-B4, which react with 95% of cases of non-T-ALL.[11,208] Second, clonal rearrangements of immunoglobulin genes provide strong evidence for the B cell lineage of most cases of non-T-ALL.[156,206,208]

Although Ia antigen is present on most non-T-ALL cells, and CALLA is present in 75% of cases of non-T-ALL, these antigens are also identified on ~10% of cases of T-ALL. Therefore, B cell-associated antigens (Table 1), which are not identified on T-ALL cells, are the most useful in distinguishing non-T-ALL. The B1 and B4 antigens are model antigens for this discussion.

Less than 5% of cases of ALL express SmIg (usually IgM); these cells are typically classified as B-ALL. These cells generally express other B cell antigens, including B1 (CD20), B4 (CD19), and Ia. B-ALL in children is probably a leukemic phase of non-Hodgkin's or Burkitt's lymphoma.[193,194] Another marker that identifies a subset of non-T-ALL is Cμ heavy chain; κ and λ light chains and SmIg are typically absent.[197] These cells are considered pre-B cells. As

indicated, most cases of non-T-ALL cells of B lineage; thus $C\mu$ is useful in determining the level of differentiation. Pre-B cells that synthesize μ heavy chain are more mature than those pre-B cells that do not synthesize μ heavy chain, but are less mature than those with SmIg.

Nadler and co-workers[208] recently classified 138 patients with non-T-ALL. They divided these cases into four major subgroups. The first subgroup was Ia antigen positive, representing 5% of cases. Another subgroup expressed the Ia and B4 antigens, representing 15% of cases. The third subgroup expressed the Ia, B4, and CALLA antigens, comprising one third of the cases. Finally, one half of the cases of non-T-ALL were Ia, B4, CALLA, and B1 positive. The fourth group was further subdivided into cases with and without $C\mu$. We propose that cases with $C\mu$ be placed in a separate group (group V). The final and most differentiated group, group VI, represents SmIg-positive B-ALL (Table 7).

Nadler and co-workers[208] also studied immunoglobulin gene rearrangements in cells from patients in groups II, III, and IV. Patients in group II (Ia and B4 positive) demonstrated rearranged heavy chain genes, with germ lines for both κ and λ light chain genes. Based on these data, they hypothesized that the group II phenotype represents the earliest stage in B cell maturation. Patients in group III had rearranged heavy chain genes and most had κ light chain gene recombination, either rearrangement or deletion. Patients in group IV had rearranged heavy chain genes; two of four had deletions of κ.

Virtually all non-T ALL cells have immunoglobulin heavy chain rearrangement; not all of them, however, demonstrate light chain rearrangements. Because heavy chain rearrangements also occur in non-B cells[159,160] immunoglobulin heavy chain rearrangements are insufficient to assign non-T-ALL to the B cell lineage. Nadler and co-workers have proposed that the B4 antigen provides the most important independent parameter with which to identify B cell-derived non-T cell ALL. More recently, up to 25% of non-T ALL have been reported to have rearrangements of the $T\beta$ receptor.[174] This observation emphasizes the necessity of combining DNA genotyping and surface marker analyses for more precise classification of leukemia and lymphoma. The remaining 5% of morphologically and cytochemically defined non-T cell ALLs (group I), which express the Ia but not the B4 antigen, most likely represents the earliest stage of pre-B cell differentiation. This hypothetical scheme of early B cell differentiation is further supported by data demonstrating that all of the proposed ALL subgroups can be identified in normal fetal liver and bone marrow and in normal adult bone marrow.[208]

Table 7. Classification of Non-T-ALL

Group	Antigens				Cytoplasmic μ	Surface Membrane Immunoglobulin
	Ia	B4	CALLA	B1		
I	+	−	−	−	−	−
II	+	+	−	−	−	−
III	+	+	+	−	−	−
IV	+	+	+	+	−	−
V	+	+	+	+	+	−
VI	+	+	+/−	+	−	+

T-ALL. T-ALL represents 15% to 25% of cases of ALL. Clinical features associated with T-ALL include a high blast cell count, predominance of older male patients, and mediastinal masses. T-ALL was originally identified by rosetting with sheep erythrocytes. The most sensitive marker for T-ALL is probably the pan-T 40 kd antigen identified by the anti-Leu-9 antibody (CD7). This antigen is present on most thymocytes and T cells but not on non-T-ALL or B cell lymphomas or leukemias.[45-48] In a study of 23 patients with T-ALL, all cases expressed the Leu-9 antigen.[46] Although it was previously reported that anti-Leu-1 (CD5) is the most sensitive marker for T-ALL,[202,209] three of these 23 patients whose cells were anti-Leu-9 positive were negative with anti-Leu-1.[46] Anti-Leu-9 reacts with a small proportion of cases that appear to be myeloid leukemias.[210] In addition, an unusually high incidence of CALLA, Ia, and BA-2 expression has been reported in adults with T-ALL.[211] Recently, rearrangement of the $T\beta$ receptor gene in cases of T-ALL has been reported.[171-175] Two cases of T-ALL with the phenotype of early thymocytes were reported to have no rearranged $T\beta$ receptor genes, suggesting that $T\beta$ rearrangement may occur later in thymocyte development.[212]

Further subclassification of T-ALL is controversial. Reinherz and colleagues proposed a subclassification for T-ALL according to the level of thymic differentiation.[201] Several elements of their subclassification of T-ALL have been confirmed; others are controversial. The most primitive thymocytes, referred to as early or stage I thymocytes, react with T9 and T10 antibodies and account for ~10% of the thymic cells. In their study, Reinherz and co-workers reported that most T-ALL cells express antigens found on early thymocytes. The next level of thymic differentiation, which includes the majority of thymocytes, is referred to as common or stage II. These cells lose T9, retain T10, and acquire T6 (CD1), T4/Leu-3 (CD4), and T8/Leu-2 (CD8) antigens. Approximately 20% of cases of T-ALL express this phenotype. Mature stage III thymocytes no longer express T6 but segregate into T4/Leu-3 or T8/Leu-2 subsets similar to peripheral blood T lymphocytes. Only rarely did Reinherz and colleagues find T-ALL cells with the phenotype of mature thymocytes or circulating T lymphocytes. In a more recent study, Roper and coworkers[204] confirmed many of the findings reported by Reinherz and colleagues, but reported some major differences. In this study, only one third of the T-ALL patients had the phenotype of early or stage I thymocytes; most had the phenotype of either intermediate or late stage thymocytes.

In Table 8, we summarize these data and propose a scheme for the classification of T-ALL. The common marker for all of the subgroups is Leu-9. Nearly all cells also express Leu-1 and most express T11/Leu-5 (CD2) that identifies the receptor for sheep erythrocytes. Cells in subgroup I also express T9 and/or T10. The pan T antigen, identified by T3/Leu-4 (CD3), represents a mature antigen and is not found on group I cells. The T4/Leu-3 helper-associated antigen, the T8/Leu-2 suppressor-associated antigen, and the T6 antigen are not expressed on group I cells.

The next level of differentiation is group II. T9 is found on some cells;[204] however, the T6 antigen as well as the simulta-

Table 8. Classification of T-ALL

Group	Antigens						
	Leu-9*	Leu-1	T11/Leu-5	T3/Leu-4	T4/Leu-3	T8/Leu-2	T6
I	+	+ (90%)	+ (75%)	−	−	−	−
II	+	+	+	+ (25%)	+ (90%)	+ (90%)	+
III	+	+	+	+	+/−†	+/−†	−

*Found on virtually all T-ALL cells.

†No longer simultaneous expression of T4/Leu-3 and T8/Leu-2 as found in group II.

neous expression of T4/Leu-3 and T8/Leu-2 antigens clearly distinguish group II from group I. Some cells in group II may also express T3/Leu-4. Group III T-ALL cells lose the T6 antigen and segregate into cells that have the phenotype of mature thymocytes and T lymphocytes (T3/Leu-4, T4/Leu-3 or T3/Leu-4, T8/Leu-2).

Although Roper and co-workers[204] searched for clinical correlations among these three groups of T-ALL, they found no unique clinical features among the subgroups and no differences in remission duration or survival. However, the groups were too small for statistically valid conclusions. Presently, we believe it useful to subclassify T-ALL using this system, so that data from a number of institutions can be analyzed for clinical correlations between the subgroups of T-ALL (Table 8).

Non-Hodgkin's lymphoma. The non-Hodgkin's lymphomas are a diverse group of neoplasms whose pathologic classification is controversial. It is even more difficult to correlate pathologic classification with immunologic classification. Several immunologic patterns emerge, however, and we will attempt to place them within the non-Hodgkin's lymphoma working classification[213] as well as the Rappaport classification.[214,215]

Follicular or nodular lymphomas. The follicular or nodular lymphomas most likely represent neoplastic proliferation of lymph node-derived follicular center B lymphocytes. The cell type may be a small cleaved cell (nodular lymphocytic poorly differentiated lymphoma by the Rappaport classification), mixed small cleaved and large cleaved or noncleaved cells (nodular mixed), or predominantly large cell (nodular histiocytic). The first two cell types fall within the working classification as low-grade lymphoma, whereas the latter cell type is classified as an intermediate-grade lymphoma. Although the predominantly small cleaved cell will almost always express high-density monoclonal SmIg, larger cells may be SmIg negative.[216,217] However, the small cleaved and large cells will routinely express Ia, B4, and B1 antigens and will often express the B2 antigen.[217] More than half of these cases will also express CALLA.[217,218] Follicular lymphoma cells may be found in the peripheral blood as a "leukemic" phase of the disease (formerly referred to as lymphosarcoma cell leukemia). These cells can usually be differentiated from chronic lymphocytic leukemia (CLL) cells because they may express CALLA, which is not expressed on CLL cells; they do not express the T1/Leu-1 pan-T antigen found on CLL cells; and they generally will have a low percentage of mouse erythrocyte rosette formation (see below).[219,220]

Malignant lymphoma, small lymphocytic. Malignant lymphoma, small lymphocytic (diffuse lymphocytic well-differentiated lymphoma in the Rappaport classification) is a low-grade malignancy, and some cases may be identical to CLL. Also included within this subclassification are the plasmacytoid lymphocytic subgroups with and without an IgM monoclonal gammopathy; some of these cases are similar to Waldenström's macroglobulinemia (described below). Surface markers on these small lymphocytic cells include low-intensity SmIg, mouse erythrocyte receptors, C'3 and receptors for the Fc portion of IgG, and Ia, B1, B2, B4, BA1, and other B cell antigens. These features are similar to CLL, and the cells also express the T1/Leu-1 pan-T antigen.

Malignant lymphoma, diffuse small cleaved cell and diffuse mixed small and large cell. Malignant lymphoma, diffuse small cleaved cell (diffuse lymphocytic poorly differentiated lymphoma in the Rappaport classification) is an intermediate prognostic group. The cells are B lymphocytes that (similar to follicular lymphoma cells) usually display large amounts of monoclonal SmIg. Unlike follicular lymphoma cells, however, they do not usually express CALLA.[218] Similar to follicular lymphoma cells, they do not express the T1/Leu-1 antigen as do cells from most small lymphocytic lymphomas and CLL. However, all these cell types have in common the expression of Ia, B4, B1, B2, and other B cell antigens.[217]

The diffuse mixed small and large cell (diffuse mixed lymphocytic-histiocytic) lymphomas have not been extensively studied but are most likely predominantly B cell diseases. They are also considered an intermediate-grade prognostic group.

Malignant lymphoma, diffuse large cell and large cell immunoblastic. In the working classification, the diffuse large cell lymphomas are considered within the intermediate prognostic group, whereas large cell immunoblastic lymphoma is a high-grade malignancy. By the Rappaport classification, both of these cell types would be described as histiocytic. This is clearly a misdesignation since 80% to 90% of cases represent clonal expansions of malignant B cells.[217,221] A high percentage of these cells express T9 and T10 antigens.[216] Fifty-seven cases of diffuse large cell lymphoma were recently studied and divided into the following subgroups: (a) B1, B4, and SmIg positive; B2 negative (50%); (b) B1, B4, SmIg, and B2 positive (30%); (c) B1 and B4 positive; SmIg and B2 negative (10%); and (4) B1 and SmIg positive, and B2 negative (10%).[222] These data suggest that most of these lymphomas represent the malignant